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Uncovering the roles of SUMOylation in Pathogenesis and Plant Defence

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Contents

Declaration

I hereby declare that the work presented here is my own and has not been submitted in any form for any degree at this or any other university.

Saad Imran Malik

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Abstract

SUMOylation is a post-translational modification in which the small ubiquitin-like protein SUMO is attached to lysine residues of the target protein. In plants, wide-spread SUMOylation is observed upon a variety of different stress cues. We tested *Arabidopsis* SUMOylation machinery knockout mutants for impaired disease resistance against *Pseudomonas syringae* pv. *tomato* (*Pst*) and identified *sumo2-1, sae1a-1 and sae2-3* as showing moderate but statistically insignificant resistance. *sumo2-1* also exhibited slightly reduced HR compared to the wild-type plants after *Pst*DC3000(*avrB*) challenge. Change in the cellular redox status is an important outcome of attempted pathogen ingress. Therefore, we also looked at the redox regulation of SUMOylation both *in vivo* and *in vitro*. We found a significant increase in SUMO1/2 conjugation and free SUMO1/2 accumulation in *atgsnor1-3* plants after *Pst*DC3000(*avrB*) challenge which was reversed during the establishment of disease in the absence of an AvrB, suggesting an important role of *S*-nitrosylation in modulating plant SUMOylation. High basal level of high molecular weight (HMW) SUMO1/2 conjugates was also apparent in *atgsnor1-3* plants even in the absence of pathogens. The changes in SUMO3 and SUMO5 remained less significant and their regulation was found to be independent of GSNOR. Biotin switch technique was employed to test further if SUMO enzymes are modified by NO. It was found that SCE1 and SAE1a are *S*-nitrosylated *in vitro* in a GSNO dose dependent fashion. MS analysis and site directed mutagenesis revealed Cys^{139} in SCE, and Cys^{93}, Cys^{158} and Cys^{231} in SAE1a as the targets of *S*-nitrosylation. We established that GSNO treatment to SCE1 differentially regulates *in vitro* SUMOylation of the model substrate *Sc*PCNA. Furthermore, the *S*-nitrosylation of Cys^{139} of SCE1 is important in fine-tuning protein SUMOylation under changing cellular redox tone. These data highlight the complexity of cross-communication between two different post-translational modifications (i.e. *S*-nitrosylation and SUMOylation) in the control of protein function.

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List of Abbreviations

3D	Three-dimensional
ABA	Abscisic acid
amiRNA	Artificial microRNA
atgsnor1-3	*Arabidopsis thaliana* GSNO reductase mutant
Avr	Avirulence/avirulent
CC	Coiled-coil
cfu	Colony forming units
Col-0	*Arabidopsis* accession Columbia
Cys or C	Cysteine
CysNO	*S*-nitrosocysteine
dpi	Days post-infiltration
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDS1	Enhanced disease susceptibility 1
EF-Tu	Elongation factor Tu
EIX	Ethylene inducing xylanase
ESD4	Early in short days 4
EST	Expressed sequence tag
ET	Ethylene
ETI	Effector-triggered immunity
GSH	Glutathione
GSNO	*S*-nitrosoglutathione
GSNOR	*S*-nitrosoglutathione reductase
HEK293	Human embryonic kidney 293
HMW	High molecular weight
hpi	Hours post-infiltration
HR	Hypersensitive response
JA	Jasmonic acid
kD	Kilo Dalton
KO	Knockout

LC	Liquid chromatography
LRR	Leucine-rich repeats
Lys or K	Lysine
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen activated protein kinase
MMTS	Methyl methanethiosulfonic
MS	Murashige and Skoog
MS	Mass-spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NahG	Salicylate hydroxylase gene
NBS	Nucleotide binding site
NF-κB	Nuclear factor Kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
NPR1	Non-expressor of *PR1* genes
NR	Nitrate reductase
PAD4	Phytoalexin deficient 4
PAGE	Polyacrylamide gel electrophoresis
PAL	Phenylalanine ammonia lyase
PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell death
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PR	Pathogen related
PRR	Pattern recognition receptor
Pst	*Pseudomonas syringae* pv. *tomato*
PTI	PAMP-triggered immunity
PTM	Post-translational modification/modifier
R	Resistance
RBOH	Respiratory burst oxidase homolog
RLK	Receptor-like kinase
RNS	Reactive nitrogen species

ROI	Reactive oxygen intermediate
ROS	Reactive oxygen species
RT	Reverse transcriptase
SA	Salicylic acid
SABP3	Salicylic acid binding protein 3
SAE	SUMO activating enzyme
SAG	Glucose-conjugated SA
SAR	Systemic acquired resistance
Sc	*Saccharomyces cerevisiae* (Yeast)
SCE1	SUMO conjugating enzyme 1
SDS	Sodium dodecyl sulphate
Ser or S	Serine
SIM	SUMO interacting motif
SNO	*S*-nitrosothiol
SP-RING	Siz-PIAS ring
SUMO	Small ubiquitin-like modifier
TTSS	Type-III secretion system
TAIR	The *Arabidopsis* Information Resource
TB	Trypan blue
TIR	Toll-interleukin 1 receptor
TLR	Toll-like receptors
T-SUMO	Tomato SUMO
Ub	Ubiquitin
Ubc9	Mammalian SUMO conjugating enzyme
ULP	Ubiquitin-like protease
UTR	Untranslated region
WT or wt	Wild-type
XopD	*Xanthomonas* outer protein D
YopJ	*Yersinia* outer protein J

Chapter 1

1 Introduction

1.1 Plants, Pathogens and Diseases

Plants are exposed to hundreds of different types of pathogens in different regions, climates and seasons. However, they are predominantly resistant to these intruders and only a few are capable of causing disease; one of the outcomes of plant-microbe interaction. In spite of the fact that disease is an exception not a rule, plant diseases have had catastrophic effects on human living in the past resulting in hunger, famines, displacements and deaths, in addition to enormous economic losses. Policy-makers have now taken up the issue of food security under changing global environments where plant disease resistance holds a fundamental role. Most of us would agree to what Sir David Baulcombe said in the February 2010 editorial of *Science* "*The world must produce 50 to 100% more food than present under environmental constraints that have not applied in the past.*"

Plant diseases are one of the biggest challenges faced by plant biologists today. A great deal of success has been achieved with pesticides, but environmental and health concerns and high costs frame us into a more thoughtful approach of exploiting the innate defence system and tailoring the plant genes into novel combinations of disease resistance pathways. Conventional breeding and selection have been, and are still, the most popular and reliable methods of achieving disease resistance over centuries without knowing the underlying genetic or molecular basis. It was in the 1942, that H. H. Flor published the first

evidence of genetic interactions of resistance in the host and virulence in pathogen (Flor, 1942, 1955). These findings laid the foundation of our understanding of the genetic and biochemical basis of plant disease resistance. A vast increase in our knowledge especially in the past two decades resulted in a paradigm shift from breeding to engineering of disease resistance in plants. However, the complex interactions during the multiple signalling pathways and characterization of multi-genes undertaking these events is still lagging behind and much is to be uncovered yet.

1.1.1 Plant Innate Immunity

Plant pathogens depend on the plants for their nutriment needs which they get either after killing the plants (necrotrophs), or may require a living host to complete their life cycle (biotrophs) while they feed on them, whilst the host plant eventually dies. To successfully invade the host plant, a pathogen must first be able to circumvent the chemical and/or mechanical barriers. These are waxy cuticles, rigid cell walls and anti-microbial toxins produced by the plant to prevent intrusion. In addition to these pre-formed barriers, plants possess a very sophisticated immune system capable of detecting any invasion and responding to it effectively. Plant innate immunity can be broadly classified into two categories; PAMP-triggered immunity (PTI) classically known as basal or horizontal resistance and effector-triggered immunity (ETI) also known as *R* gene, gene-for-gene, race specific or vertical resistance (Jones and Dangl, 2006; Bent and Mackey, 2007; Gohre and Robatzek, 2008; Boller and Felix, 2009).

1.1.1.1 PAMP or MAMP-triggered immunity

The first layer of defence comprises of pattern recognition receptors (PRRs) present at the plant's cell surface similar to the mammalian Toll-like receptors (TLR) (Hayashi et al., 2001; Staskawicz et al., 2001; Nurnberger et al., 2004; Boller and He, 2009; Takeuchi and Akira, 2010). These PRRs are capable of perceiving highly conserved generic structures in pathogens called pathogen/microbe-associated molecular patterns (PAMPs or MAMPs) also known as general elicitors (Zipfel et al., 2004; Boller and He, 2009). PAMPs or MAMPs are inevitable for microbial lifestyles hence their chances to escape recognition by the plant receptors or PRRs become very narrow (Gohre et al., 2008). When a PAMP is autonomously recognized by an individual cell's PRR proteins, systemic signals activate plant defences in the naïve tissues for any anticipated pathogen ingress. This leads to a rapid ion influx, generation of reactive-oxygen species (ROS), activation of the mitogen-activated protein kinase (MAPK) signalling cascade, hormone signalling, accumulation of phytoalexins, callose deposition, and cell wall appositions leading to resistance known as PTI (Asai et al., 2002; Jones and Dangl, 2006; Zipfel, 2008; Clay et al., 2009). PTI is a relatively weak immune response but is effective in preventing microbial growth and keep them confined to the intercellular spaces (apoplasts) away from the cytosolic nutrients (Takken and Tameling, 2009).

Many PAMPs have been isolated form bacterial and fungal pathogens. These include peptide flg22, cold shock proteins, lipopolysaccharides or bacterial elongation factor Tu (EF-Tu) (Felix et al., 1999; Felix and Boller, 2003; Kunze et

al., 2004), fungal chitin and ergosterol (Baureithel et al., 1994; Granado et al., 1995; Kaku et al., 2006). It should be taken into account that the defence activation is triggered in minutes after PAMP perception. This is important for the plants to effectively contain pathogens when they are small in number before the bacterial titre builds up to the extent that it may overcome host PTI (Felix et al., 1999; Zipfel et al., 2004; Hann and Rathjen, 2007; Zipfel, 2009b).

The PAMPs are detected by a leucine-rich repeats – receptor-like kinase (LRR-RLK) class of proteins present at the plant cell surface which constitute PRRs. An eminent example of PAMP is flg22 which is a highly conserved, 22 amino acid long subunit of bacterial flagellum (Felix et al., 1999). The flg22 is recognized by a transmembrane flagellin receptor protein FLS2 (flagellin sensing 2) which comprises an extracellular leucine-rich repeats (LRR) ligand-binging domain, a transmembrane region and a cytoplasmic receptor-like kinase (RLK) domain (Felix et al., 1999; Gomez-Gomez and Boller, 2000; Takai et al., 2008). FLS2 makes a complex with BAK1 (BRI1-associated kinase 1) sparking a PAMP induced MAP kinase cascade leading to the activation of WRKY transcription factors which result in defence activation (Chinchilla et al., 2007; Heese et al., 2007; Pitzschke et al., 2009). Certain phytopathogenic bacteria manipulate or hijack MAP kinase signalling resulting in susceptibility. For instance a *Pseudomonas syringae* pv. *tomato* (*Pst*) effector AvrPtoB, which is an E3 ligase, facilitates ubiquitination of FLS2 when it binds to BAK1 and leads to its proteosome-mediated degradation. Consequently, downstream signalling to the MAPK module is interrupted resulting in disease (Shan et al., 2008; Pitzschke et

al., 2009). Similarly, *Arabisopsis* LysM receptor kinase CERK1 (chitin elicitor receptor kinase 1), which recognizes fungal chitin, is blocked by ubiquitin-mediated proteolysis by AvrPtoB leading to susceptibility (Gimenez-Ibanez et al., 2009).

Mutation in *FLS2* renders *Arabidopsis* more susceptible to the bacterial pathogen *Pst*DC3000 only when the bacterial suspension is sprayed on the leaf surface. However, *fls2* plants displayed susceptibility like wild-type after bacterial infiltration into the apoplast suggesting flagellin perception by FLS2 takes place at the plant cell surface (Zipfel et al., 2004). Direct binding between FLS2 and flg22 epitope was also later confirmed (Chinchilla et al., 2006).

Likewise, another highly conserved bacterial EF-Tu acts as a PAMP and is recognized by *Arabidopsis* EF-Tu receptor (EFR1) – a transmembrane LRR-receptor kinase (Zipfel et al., 2006). Particularly, the first 18 residues along the N-terminus of EF-Tu (elf18) induce oxidative burst and ethylene (ET) production in *Arabidopsis* after recognition by a PRR protein EFR1 (Kunze et al., 2004) which is a transmembrane LRR-kinase. Furthermore, *efr1* mutant plants exhibit improved T-DNA delivery by *Agrobacterium*, demonstrating its role in partial resistance of *Arabidopsis* plants against crown-gall disease (Zipfel et al., 2006).

Taking fungal pathogens into account, the activity of a fungal cell wall degrading enzyme polygalacturonase is inhibited by a plant LRR protein polygalacturonase-inhibiting protein (PGIP) which limits the pathogenicity by triggering defence responses (Di Matteo et al., 2006). This implies that the enzyme activity is inhibited by the LRR directly. A receptor protein CEBiP (chitin

elicitor binding protein) isolated from rice cell cultures (Kaku et al., 2006) and a receptor-like kinase CERK1 in *Arabidopsis* (Miya et al., 2007) was shown to play an important role in PAMP perception and defence signal transduction after fungal chitin perception at the plasma membrane.

Though PTI renders a very effective defence, there are some exceptions to PAMP recognition. Some phytopathogenic bacteria mask their PAMPs or shed their flagella during infection which prevent them from being recognised by the host receptors. Others have different amino acid composition in their flagellin domains which allows them to escape host detection (Pfund et al., 2004; Andersen-Nissen et al., 2005; Sun et al., 2006). Moreover, PTI can subsequently be restrained by the effector molecules injected directly in the host cells which may target, for example, MAPKs signalling cascade (Asai et al., 2002; Zhang et al., 2007; Zipfel, 2009a) or suppress miRNA (microRNA) silencing resulting in susceptibility (Navarro et al., 2008). Hence, the warfare between plants and pathogens does not conclude here. Pathogens have to overcome a stronger defence line called 'Effector triggered immunity' or ETI before successful invasion.

1.1.1.2 Effector-triggered immunity (ETI)

PTI offers only basal defence and may only initially stop or limit the extent of disease development or the establishment of infection (Dangl and Jones, 2001; Jones and Dangl, 2006). ETI is accomplished by a plethora of R (resistance) proteins in the host cell which recognize pathogen's avirulent (Avr*)* proteins (Staskawicz et al., 1984; Dong et al., 1991) called 'Effectors'. Gram negative bacteria are capable of deploying effector proteins directly into the host cell via

specialized syringe-like proteinaceous channels called type-III secretion system (TTSS) (Baker et al., 1997; Nimchuk et al., 2003; Mudgett, 2005; Cui et al., 2009). Effector delivery in the host's cell has two possible outcomes. Firstly, if an effector goes unchecked in the absence of a corresponding R protein, the defence response is suppressed, the pathogen becomes capable of infection and the plant susceptible; an idea was introduced by Flor in 1971. This compatible interaction leads to susceptibility and eventually disease.

Secondly, if the effector molecule is recognized by an R protein, directly or indirectly, an abrupt defence response called ETI is triggered within hours in the host cells leading to the activation of a battery of signal transduction cascades arresting pathogen growth (Staskawicz et al., 1995; Yang et al., 1997; Dangl and Jones, 2001; Gohre and Robatzek, 2008). This response is often associated with visible necrosis of plant cells at the site of infection; the consequence of a suicidal reaction known as the hypersensitive response (HR) characterized by the cell death of infected tissues (Greenberg and Yao, 2004; Romer et al., 2007). Activation of a subset of pathogenesis-related (PR) genes, calcium and ion influx, production of ROS and reactive nitrogen species (RNS), and extracellular oxidative burst are the characteristics of HR (Lamb and Dixon, 1997; Delledonne et al., 1998; Loake, 2001; Nimchuk et al., 2003; Belkhadir et al., 2004). HR is one of the earliest responses after successful pathogen recognition (Torres et al., 2006). The plant is resistant, the pathogen avirulent and the interaction is incompatible.

In addition to local cell death, HR also leads to the activation of salicylic acid (SA) dependent inducible defences in systemic tissues of the plants called systemic acquired resistance (SAR) (Ross, 1961; Delaney et al., 1994; Ryals et al., 1994; Delaney, 1997). SAR is characterized by a heightened state of resistance which subsequently protects the whole plant against a broad spectrum of pathogens (Ryals et al., 1995; Durrant and Dong, 2004; Glazebrook, 2005). Thus the ETI not only prevents colonization and spread of pathogenic bacteria but also triggers and amplifies the innate defence responses in the remote tissues to prevent future invasions.

It is estimated that 1% of the *Arabidopsis* genome is dedicated to *R* gene like sequences which encode ~150 R proteins (Dangl and Jones, 2001; Nimchuk et al., 2003; Chisholm et al., 2006). Dangle and Jones (2001) classified R proteins into five categories. The largest class of R proteins is NB-LRR named after their central nueleotide-binding (NB) and C-terminal leucine-rich repeat (LRR) domains. NB-LRR proteins either have a coiled-coil (CC) or Toll and interleukin-1 receptor (TIR) domains, therefore, sub-grouped further into CC-NB-LRR and TIR-NB-LRR on the basis of distinct N-terminal domains attached to the intercellular plasma membrane (Staskawicz et al., 1995; Hammond-Kosack and Jones, 1997; Meyers et al., 1999; Meyers et al., 2005; DeYoung and Innes, 2006).

Sequence analysis of more than 40 R proteins in recent years has revealed that most of them belong to NB-LRR class of proteins (Lukasik and Takken, 2009). Some well characterized *Arabidopsis* NBS-LRR proteins include RPS2, RPS5 and RPM1 conferring resistance to *Pst* effectors AvrRpt2, AvrPphB and

AvrRpm1/AvrB respectively (Bent et al., 1994; Chisholm et al., 2006). Similarly, a recently reported TIR-NBS-LRR protein ADR2 (activated disease resistance 2) conveys resistance against several biotrophs but not necrotrophs in *Arabidopsis* (Aboul-Soud et al., 2009). Two smaller classes of R proteins have an extracellular LRR domain which acts as a receptor for extracellular pathogen-derived ligands, and can be distinguished by the presence (e.g. in rice Xa21 which confers resistance to *Xanthomonas campestris*) or absence (e.g. in tomato Cf-2, 4, 5 or 9: which confers resistance to leaf mold fungus *Cladosporium fulvum* effectors Avr2, 4, 5 and 9, respectively) of a cytoplasmic Kin domain (Song et al., 1995; Dixon et al., 1998; Dixon et al., 2000; Fritz-Laylin et al., 2005; Chisholm et al., 2006). There is one class which has a trans-membrane signal anchor and a putative intercellular CC domain. An example of such an R protein is RPW8 which offers resistance to powdery mildew in *Arabidopsis* (Xiao et al., 2001). The smallest class of R proteins only has a cytoplasmic Ser/Thr kinase catalytic domain and an N-terminal myristoylation site associated with the plasma membrane (Bogdanove and Martin, 2000; Martin et al., 2003). This class has a single but well studied member 'Pto' (for *Pseudomonas syringae* pv. *tomato*) which confers resistance against AvrPto and AvrPtoB effector proteins. Notably, evidence for a physical interaction between these R/Avr proteins has also been found (Tang et al., 1999; Kim et al., 2002; Abramovitch et al., 2003; Xing et al., 2007).

Individual strains of gram negative bacterial pathogens like *Pseudomonas* and *Xanthomonas* can directly deploy dozens of effector proteins into the host

cytoplasm via TTSS, while fungi and oomycetes are believed to make use of haustoria or extrahaustorial matrix for their effector delivery (Chang et al., 2005; Chisholm et al., 2006; Birch et al., 2008). TTSS is encoded by a cluster of *hrp* (hypersensitive response and pathogenicity) genes present within the bacterial genomes (Bogdanove et al., 1996; Cornelis and Van Gijsegem, 2000; Mudgett, 2005). These *hrp* genes are indispensible for bacterial pathogenicity. Phytopathogenic bacteria disarmed in *hrp* (*hrp* $^{-}$*)* are unable to cause disease on susceptible host or even HR on otherwise resistant plants (Lindgren, 1997; Bent and Mackey, 2007).

How R/Avr proteins interact to induce defence remained a very important question. Intense efforts with several R and Avr proteins could only reveal two instances of physical interaction. First, a Pto and AvrPto or AvrPtoB interaction reported in *Arabidopsis* mentioned above. Second, a rice TIR-NBS-LRR protein RRS1-R interaction with PopP2 (a bacterial avirulent type-III effector) which confers resistance against bacterial wilt (Deslandes et al., 2003). A more thoughtful approach was hypothesized by van der Biezen and Jones in 1998 known as the 'guard hypothesis' which states that R proteins physically interact with plant proteins (guardees) targeted or modified by the pathogen *avr* gene products and result in the activation of defence response (Van der Biezen and Jones, 1998; McDowell and Woffenden, 2003; Jones and Dangl, 2006). However, how the LRR domain recognizes an effector remains unclear (Takken and Tameling, 2009).

1.1.2 Signalling Networks in Plant Defence

The signalling networks involved in defence activation depend largely on the type of phytopathogen and primarily rely on three signalling molecules i.e. salicylic acid (SA), jasmonates (JAs) and ethylene (ET). SA is an important signal both in PTI and ETI during biotrophic or hemi-biotrophic pathogen infection and is required to trigger HR. The role of ET and JA is more inclined towards resistance against necrotrophic pathogens. JA signalling is primarily involved in wounding and herbivory. However, JA in collaboration with ET is known to activate defences against necrotrophs. Interestingly, the plants can either activate JA or SA signalling but not both. There has been a considerable cross-talk between these different branches and both synergistic and antagonistic interactions have been reviewed in many reports (Dong, 1998; Kunkel and Brooks, 2002; Glazebrook, 2005; Wiermer et al., 2005; Grant and Lamb, 2006; Loake and Grant, 2007; Grant and Jones, 2009).

SA accumulation in infected tissues after pathogen recognition underpins its central role in plant defence signalling. Many-fold increases in SA levels have been reported not only at the site of infection during HR but also in the remote tissues after pathogen challenge leading to the activation of SAR (Malamy et al., 1990; Metraux et al., 1990; Delaney et al., 1994; Conrath et al., 1995). Exogenous application of SA or SA analogues e.g. benzothiadiazole also induces SAR even in the absence of pathogens (Lawton et al., 1996; Dong, 2004). However, SA is unlikely to be a mobile signal but is important in signal transduction required for SAR induction (Rasmussen et al., 1991; Vernooij et al., 1994; Ryals et al., 1995;

Loake and Grant, 2007). Transgenic *Arabidopsis* plants expressing *NahG*, a bacterial salicylcate hydroxylase gene which encodes an enzyme that metabolises SA to catechol, do not accumulate SA. In the *NahG* transgenic lines, the activation of SAR was compromised, *PR* gene expression abolished and the plants became susceptible to biotrophs (Gaffney et al., 1993; Lawton et al., 1995). However, it was established later that *NahG* expressing plants also have low levels of camalexin (a phytoalexin) besides SA and the disease phenotype may not be merely due to the low SA contents (Heck et al., 2003). Likewise, NPR1 (non-expressor of *PR* genes) is a positive regulator of SAR and functions downstream of SA. *npr1* null-mutants are insensitive to exogenous SA treatment, fail to express *PR* genes and are compromised in basal resistance (Cao et al., 1997; Dong, 2004; Tada et al., 2008).

Mutants deficient in SA (*sid2* – SA induction deficient 2) are susceptible to biotrophs and not necrotrophs while mutants deficient in JA signalling (*coi1* – coronatine insensitive 1 or *jar1* – jasmonic acid resistant 1) or ET signalling (*ein2* – ethylene insensitive 2) behave conversely. This suggests distinct signal transduction pathways for SA and JA/ET-dependent responses (McDowell and Dangl, 2000; Glazebrook, 2001, 2005; Santner and Estelle, 2009). *EDS1* (enhanced disease susceptibility 1) and its counterpart *PAD4* (phytoalexin deficient 4) (Glazebrook et al., 1996) are required by TIR-NBS-LRR class of R proteins for SA accumulation and disease resistance (Jirage et al., 1999; Hu et al., 2005). Moreover, *eds1* and *pad4* mutants accumulate less ROI as compared to wild-type in a non-host pathogen *Blumeria graminis f.* sp. *tritici* (*Bgt*) infections

(Yun et al., 2003). Nevertheless, CC-NBS-LRR signalling is independent of *EDS1-PAD4* but requires NDR1 (non-race-specific disease resistance 1) and *PBS2* (*avrPphB* Susceptible 1) (Aarts et al., 1998; Feys et al., 2001; Glazebrook, 2001; Wiermer et al., 2005). Furthermore, the JA insensitive *coi1* mutant is blocked in *PDF1.2* (plant defensin 1.2) gene expression and is highly susceptible to a necrotroph *Alternaria brassicicola* (Penninckx et al., 1998) while *ein2* mutant plants are more susceptible to *Botrytis cinerea*. *PDF1.2* expression requires both JA and ET, simultaneously, but is independent of SA or NPR1 (Thomma et al., 1998; Thomma et al., 1999; Glazebrook, 2005). Recently, azelaic acid, a nine-carbon dicarboxylic acid isolated from the *Arabidopsis* vascular sap, was also found to prime SA induced systemic immunity (Jung et al., 2009).

1.1.3 Oxidative Burst and Cell death

Rapid generation of reactive oxygen species (ROS) like superoxide anion (O_2^-), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2) and singlet oxygen (O_2), collectively known as the oxidative burst, is one of the earliest events during ETI which leads to the hypersensitive cell death (Apostol et al., 1989; Levine et al., 1994; Lamb and Dixon, 1997; Grant and Loake, 2000; Grant et al., 2000a; Greenberg and Yao, 2004). Under perturbed physiological conditions like pathogen ingress, the imbalance between ROS production and scavenging leads to high ROS accumulation which potentiate an irreversible damage to the plant tissues (Apel and Hirt, 2004).

ROS production is mediated by an increase in cytosolic Ca^{2+} through extracellular Ca^{2+} influx via the plasma membrane resulting in downstream

activation of calmodulin and protein kinases (Grant et al., 2000b; Ma and Berkowitz, 2007; Du et al., 2009). A cytosolic membrane-bound respiratory burst oxidase (RBO), that is, NADPH oxidase is the source of reactive oxygen intermediates (ROI) accumulation and ROS production in mammals which converts O_2 to O_2^- (Doke, 1983; Lamb and Dixon, 1997; Torres and Dangl, 2005). The superoxide dismutase (SOD) converts O_2^- to H_2O_2 which can give rise to •OH. In *Arabidopsis*, ROS production in the apoplast is implicated by a family of respiratory burst oxidase homolog (*At*rboh) genes which closely resemble mammalian $gp91^{phox}$ subunits of NADPH oxidase (Torres et al., 2002; Torres et al., 2005).

HR is a genetically controlled phenomenon, however, the underlying mechanism regarding HR in relation to ROS production is more or less obscure. Upregulation of *RBOH* genes during pathogen interactions was evident (Desikan et al., 1998; Yoshioka et al., 2001) before genetic evidence proved that loss-of-function mutation in *atrbohD* and *atrbohF* exhibit reduced pathogen induced ROI accumulation and cell death in response to avirulent bacterial pathogens. These genes seem to work together to produce ROIs (Torres et al., 2002; Yoshioka et al., 2003). The *atrbohD* null-mutation suppressed the production of reactive oxygen intermediates (ROIs) while *atrbohF* mutant has attenuated HR. (Torres et al., 2002). Furthermore, *dnd1* plants induce normal defence responses against both virulent and avirulent pathogens but are defective in hypersensitive cell death (Clough et al., 2000) suggesting bacterial growth can be restricted even in the absence of cell death. These findings imply that cell death is not merely an

outcome of ROI production but is a genetically controlled process (Yu et al., 1998). Recently, Hatsugai and co-workers reported that fusion of the large central vacuole with the plasma membrane results in an extracellular discharge of antibacterial compounds in order to contain pathogen growth along with cell death induction (Hatsugai et al., 2009).

After recognizing the role of caspases (for cysteine-dependent aspartate-specific proteases) in animal apoptosis (Thornberry and Lazebnik, 1998; Nicholson and Thornberry, 2003), growing evidence suggest that caspase-like proteins or metacaspases are critical for the execution of cell death in plants (Gilroy et al., 2007; Bonneau et al., 2008), but no true caspase has been found (Bozhkov et al., 2005; verHe et al., 2008). A small family of functionally redundant cysteine protease genes *CATB* (cathepsin B) was shown to encode caspase-like proteins. They were found to be involved in cell death and disease resistance in plants against non-host pathogens after virus-induced gene silencing (VIGS) or using cathepsin B inhibitors in tobacco plants (Gilroy et al., 2007; McLellan et al., 2009). Suppression of hypersensitive cell death, initiated by TIR-NB-LRR proteins through EDS1, was evidenced in autophagy-deficient mutants *atg* (autophagy-related) suggesting autophagy also contributes to HR development (Liu et al., 2005; Hofius et al., 2009). Interestingly, use of cathepsin inhibitors on *atg7* mutants resulted in significant reduction in cell death suggesting different but overlapping pathways are invoked in plants by different phytopathogens (Hofius et al., 2009). Recently, a novel plant phytaspase (plant aspartate-specific protease)

has been reported as essential for PCD in tobacco against tobacco mosaic virus (TMV) (Chichkova et al., 2010).

Several *Arabidopsis* mutant screens led to the identification of mutations which show uncontrolled cell death even in the absence of pathogens. Such mutants called 'lesion memics' include *acd2* (accelerated cell death 2) (Greenberg and Ausubel, 1993) and *lsd1* (lesions simulating disease 1) (Dietrich et al., 1994). The HR once initiated in *lsd1* at a small site spreads unrestricted to the entire leaf a phenomenon called 'runaway cell death' (RCD) and is also associated with O_2^- accumulation (Jabs et al., 1996). Moreover, *LSD1* is a negative regulator of cell death in plants (Dietrich et al., 1997), functions in an SA dependent fashion (Aviv et al., 2002) and engages the *EDS1/PAD4* signalling cascade (Loake, 2001; Rusterucci et al., 2001). In *acd2* plants, the lesion formation leading to RCD can be triggered by a bacterial toxin coronatine and is due to the accumulation of chlorophyll breakdown products which seems to amplify disease symptoms (Mach et al., 2001; Pruzinska et al., 2003). An ankyrin-repeat protein ACD6 (accelerated cell death 6) was found to be involved in SA dependent defence responses against bacterial pathogens and *acd6* mutant *Arabidposis* plants undergo cell death even in the absence of pathogens (Lu et al., 2003)

The ROS mediated activation of MAPK cascade and subsequent defence activation is now well documented (Grant et al., 2000a; Kovtun et al., 2000; Laloi et al., 2004). ROS are also related to the activation of SAR in an SA dependent fashion (Chamnongpol et al., 1998). H_2O_2 accumulation and a transient microburst in the remote tissues following the oxidative burst in the inoculated

leaves suggest that ROS might act as a second messenger for the activation of SAR (Alvarez, 2000; Neill et al., 2002; Durrant and Dong, 2004; Fobert and Despres, 2005). A striking feature of necrotrophic pathogens e.g. *Botrytis cinerea* and *Sclerotinia sclerotiorum* is the induction of oxidative burst for their own advantage in order to kill the infected tissues and extract nutrients (Govrin and Levine, 2000). Hence, oxidative burst and cell death protects plants against biotrophs only. Necrotrophic pathogens, however, are capable of exploiting this process for their own benefit in order to aid colonization. Along with ROS production, reactive nitrogen species (RNS) play a vital role in defence activation and a balance between ROS and RNS is critical for fine-tuning defence responses apart from growth and development.

1.1.4 Nitric Oxide in Defence Signalling

Nitric oxide (NO) is a gaseous free radical orchestrating a variety of cellular activities (Wendehenne et al., 2001). Being small in size, least charged, stable and diffusible with a half-life of a few seconds, NO is regarded as a key signalling molecule both in animals and plants. NO emerged as an important molecule in mammals first where its role was recognized in smooth muscle relaxation, neurotransmission, immune response and platelet aggregation (Murad, 1986; Schmidt and Walter, 1994; Nathan, 1995; Tuteja et al., 2004). Long after the discovery of NO generation in plants in 1979 (Klepper, 1979), we began to appreciate its pivotal role in plant biology including growth, development, reproduction and stress responses (Delledonne et al., 1998; Durner et al., 1998;

Beligni and Lamattina, 2000; Lamattina et al., 2003; He et al., 2004; Besson-Bard et al., 2008; Hong et al., 2008).

NO synthesis in animals and plants mainly takes place by the conversion of L-arginine to citrulline (Wendehenne et al., 2001; Corpas et al., 2009). In animals, a small family of nitric oxide synthases (NOS) catalyse this conversion and require oxygen and NADPH (Wendehenne et al., 2001). In plants, however, the mechanism underlying NO synthesis is still elusive and no *NOS*-like gene has been identified thus far. NOS-like activity in *Arabidopsis* was biochemically related to a mammalian orthologue *AtNOS1* (Guo et al., 2003; Zeidler et al., 2004; Guo and Crawford, 2005). Despite *atnos1* plants have reduced endogenous NO levels, *At*NOS1 has been designated a cGTPase (Moreau et al., 2008) and significantly was found not to be directly involved in NO biosynthesis (Zemojtel et al., 2006). In response to this controversy, *AtNOS1* was renamed *AtNOA1* (nitric oxide associated 1) (Crawford et al., 2006). Other proposed routes for NO production in plants include non-enzymatic reduction of apoplastic nitrite ions (NO_2^-) to NO (Bethke et al., 2004) or nitrate reductase (NR)-dependent NO synthesis using NADPH as an electron donor (Yamasaki, 2000).

In mammalian inflammatory response, phagocytes produce large amounts of NO and O_2^- which react to produce a highly reactive and diffusible adduct peroxyinitrite ($ONOO^-$) leading to neurotoxicity (Bonfoco et al., 1995; Nathan, 1995; Marla et al., 1997). In plants, ROIs along with NO production are the important outcomes of an incompatible pathogen interaction (Delledonne et al., 1998). A balanced production of two partners is critical for cell death activation

and high NO level alone in the absence of an oxidative burst is insufficient to trigger HR (Delledonne et al., 1998; Hong et al., 2008). Unlike mammals, plants being relatively resistant to $ONOO^-$ induced toxicity are thought to initiate cell death by the reaction of NO with H_2O_2 catalysed by superoxide dismutase (SOD) (Delledonne et al., 2001). The underestimation of basal NO levels during HR was identified by measuring the trapped NO in $ONOO^-$ which might be due to elevated SOD activity following defence activation (Vanin et al., 2004; Hong et al., 2008). Moreover, the role of the H_2O_2 and O_2 partnership is further supported by catalase-deficient (CAT1AS) transgenic tobacco plants retaining as little as 10% of the wild-type catalase activity (Dat et al., 2001). Compared to wild-type, more cell death was recorded in transgenic lines following sodium nitroprusside (an NO donor) infiltration in leaves after moderate-high light exposure (Zago et al., 2006).

NO is involved in a number of defence signalling pathways including SA and JA accumulation (Huang et al., 2004), SA dependent ethylene production (Mur et al., 2008), activation of defence genes encoding *PR1* and *PAL* (phenylalanine ammonia lyase) (Grun et al., 2006), and ABA mediated stomatal closure (Desikan et al., 2004). Collectively, these findings emphasize that NO is a key player in plant defence responses.

1.1.5 NO signalling by *S*-nitrosylation

NO exerts its function by protein *S*-nitrosylation which is a reversible post-translational modification of proteins characterized by the addition of an NO moiety to a reactive cysteine thiol forming an *S*-nitrosothiol (SNO) (Stamler et al.,

1992; Hess et al., 2001; Wang et al., 2006). Protein *S*-nitrosylation was fist reported in endothelium-dependent relaxation of smooth muscles in humans in a landmark article by Stamler et al. (1992). This led to the emergence of a highly regulated redox based mechanism governing protein functions both in animals and plants. The development of the biotin switch technique (Jaffrey and Snyder, 2001) subjugated the technical limitations to assay SNOs and led to the identification of hundreds of proteins targeted by *S*-nitrosylation both in plants and animals (Jaffrey et al., 2001; Lindermayr et al., 2005; Torta et al., 2008). Identification of 63 *S*-nitrosylated proteins from cell cultures and 52 proteins from leaves in *Arabidopsis* revealed that these proteins belong to almost all major categories of the proteome participating in a wide array of cellular activities including metabolism, trafficking, stress response, cytoskeleton, signalling and photosynthesis (Lindermayr et al., 2005). Addition of mammalian targets of *S*-nitrosylation makes this list more exhaustive where *S*-nitrosylation has been shown to regulate dozens of regulatory proteins and enzymes, ion channels, metal and DNA binding proteins, and transcriptional factors (reviewed in Hess et al., 2005).

The NO moiety required for *S*-nitrosylation can be derived from different RNS (NO_x, N_2O_3, $ONOO^-$), auto-*S*-nitrosylation by metalloprotein NO-complexes and from thiol-to-thiol NO shifts called transnitrosylation (Arnelle and Stamler, 1995; Hess et al., 2005; Lindermayr and Durner, 2009). Nevertheless, *S*-nitrosoglutathione (GSNO) acts as a global reservoir of NO in cells and is the main and most widely used NO donor for *S*-nitrosylation both *in vivo* and *in vitro*

(Singh et al., 1996; Inoue et al., 1999; Jaffrey et al., 2001). The synthesis of GSNO takes place by an O_2 dependent reaction of NO with glutathione (GSH – a strong antioxidant produced in the cells) (Gaston et al., 1993; Singh et al., 1996). This is now well documented that pathophysiological conditions induce nitrosative stress both in animals and plants which results in GSH conversion to GSNO leading to protein *S*-nitrosylation (Eu et al., 2000; Gaston, 2003; Romero-Puertas et al., 2008).

Predicting *S*-nitrosylation site(s) within proteins is an important area of discussion in SNO biology. At first, an acid-base consensus motif, with flanking acidic (Asp, Glu) and basic (Arg, His, Lys) residues, was proposed (Stamler et al., 1997; Hess et al., 2005). However, its reliability was questioned by many succeeding reports. It was suggested that binding of an NO moiety to the Cys residues is an exquisitely selective process determined collectively by many factors such as accessibility of the Cys to the NO donor and the hydrophobicity or identity of the proximate residues, thiol pKa, the presence of metal ions like Mg^{2+} or Ca^{2+}, and protein tertiary structure (Lai et al., 2001; Wang et al., 2006; Marino and Gladyshev, 2009). Moreover, *S*-nitrosylation does not seem to be dependent upon the total number of Cys residues within a protein. For example, in ryanodine receptors, only one Cys (Cys^{3635}) is *S*-nitrosyled out of ~ 50 cysteines, of which 12 are flanked by an acidic residue (Sun et al., 2001). The only *S*-nitrosylated Cys is flanked by Ala and Phe which are not acidic residues.

Since *S*-nitrosylation is a reversible process, protein denitrosylation leading to SNO decomposition is another aspect of modulating protein functions.

Protein denitrosylation is a tightly regulated mechanism catalysed by two major enzyme systems, GSNO reductase (GSNOR) and thioredoxins (TRX) collectively known as denitrosylases (Benhar et al., 2008; Benhar et al., 2009). For instance, TRX-1 mediated denitrosylation of caspase-3 results in its activation to promote apoptosis in mammals (Benhar et al., 2008). Likewise, *S*-nitrosylation mediated oligomerization of a primary plant defence regulator NPR1 is subsequently catalyzed by TRX-h upon defence activation which causes it to disintegrate into a monomeric form. NPR1 monomer makes its way through the nuclear pores activating *PR1* and eventually defence responses (Mou et al., 2003; Spoel et al., 2003; Dong, 2004; Tada et al., 2008). Recently, *S*-nitrosylation of a thioredoxin-interacting protein (Txnip) was shown to repress Txnip activity, thereby facilitating TRX-mediated denitrosylation (Forrester et al., 2009). This indicates a feed-back regulation of *S*-nitrosylation by denitrosylation. Collectively, these findings suggest that protein *S*-nitrosylation and denitrosylation work in parallel to regulate protein functions.

GSNO is the major low-molecular weight SNO adduct, rapidly produced in cells. Cellular GSNO turn-over is accomplished by GSNOR to keep its homeostasis in line (Gaston et al., 1993; Liu et al., 2001; Benhar et al., 2009). GSNOR was first recognized as a glutathione-dependent formaldehyde dehydrogenase (FDH) with GSNO reductase activity controlling cellular SNO levels and was found conserved in bacteria and humans (Liu et al., 2001). Later, a homologue of this gene was found in plants and was shown as upregulated after SA treatment (Sakamoto et al., 2002; Diaz et al., 2003). GSNOR belongs to the

class III alcohol dehydrogenase (ADH) family of enzymes. Surprisingly, this enzyme neither hydrolyses alcohol nor directly acts on protein SNOs (Dolferus et al., 1997; Hess et al., 2005). Importantly, an equilibrium between GSH/GSNO is important in the cells to accomplish normal functions which is maintained by GSNOR. Loss of GSNOR function results in dysregulated *S*-nitrosylation resulting in deleterious effects on plant (Feechan et al., 2005) and animal (Liu et al., 2004) lives, which are discussed in the following section with a focus on plants.

1.1.6 *S*-Nitrosylation and Plant Diseases Resistance

The role of *S*-nitrosothiols in plant defence was first unveiled by Feechan and co-workers (Feechan et al., 2005). Loss of *Arabidopsis* GSNOR function (*atgsnor1-3*) resulted in an increase in cellular SNO levels which was further increased after *Pst*DC3000(*avrB*) challenge. *atgsnor1-3* null-mutants showed reduced SA levels, and were compromised in PTI and ETI along with normal developmental features. In contrast, the gain-of-function mutation by *AtGSNOR1* over-expression decreased the cellular SNOs and exhibited enhanced disease resistance (Feechan et al., 2005). Taken together, *At*GSNOR1 was held responsible for global GSNO turn-over and SNO homeostasis in plants and was found to be required for basal, non-host and *R* gene mediated defences (Feechan et al., 2005; Wang et al., 2006). Previous research using a mouse model showed that GSNOR deletion ($GSNOR^{-/-}$) resulted in substantial increase in total SNOs, nitrosative stress and cell damage leading to increased pathogenesis and higher mortality after septic shocks (Liu et al., 2004). In another study, $GSNOR^{-/-}$ mice

were found to be susceptible to carcinogen-induced hepatocarcinoma and displayed impaired DNA repair (Wei et al., 2010). Conversely, GSNOR$^{-/-}$ mice exhibited reduced myocardial injury under normoxic conditions as compared to wild-type mice due to *S*-nitrosylation of hypoxia inducible factor-1α (Lima et al., 2009).

Further evidence for the role of *S*-nitrosylation in plant defence came in a recent report where *S*-nitrosylation of *Arabidopsis* SA binding protein 3 (*At*SABP3) at Cys^{280} suppressed its SA binding and carbonic anhydrase (CA) activity required for defence activation after pathogen challenge (Wang et al., 2009). Moreover, *PAD2-1* (phytoalexin deficient 2-1) was reported to be involved in GSH biosynthesis in *Arabidopsis*. A *pad2-1* null-mutation resulted in reduced GSH levels and increased susceptibility to pathogens suggesting an adequate GSH level is required for the establishment of disease resistance in plants (Noctor et al., 2002; Parisy et al., 2007). Likewise, exogenous application of NO donors, cyclic GMP (cGMP) and cyclic ADP-ribose to tobacco plants triggered *PR1* gene expression and phenylalanine ammonia lyase (PAL) synthesis (Durner et al., 1998), which acts as a precursor for synthesis of certain defence related compounds, particularly SA (Mauch-Mani and Slusarenko, 1996). In a random screen of proteins *S*-nitrosylated during HR in *Arabidopsis*, 16 *S*-nitrosylated proteins were identified by using 2D gel electrophoresis coupled with mass spectrometry (MS) (Romero-Puertas et al., 2008). On the whole, these findings underpin the importance of *S*-nitrosylation / denitrosylation in the regulation of

protein function and defence activation and highlight the importance of GSNOR in the regulation of GSH and GSNO equilibrium.

1.2 SUMOylation

SUMOylation is a reversible post-translational modification of proteins, biochemically similar to ubiquitination but functionally distinct. Ubiquitin (Ub) being named for its ubiquitous presence in eukaryotic cells, was first discovered as a small polypeptide (76 residues) in mid-1970s (Goldstein et al., 1975). A few years later, Hershko and co-workers described the ubiquitin-mediated proteolysis by the 26S proteosome (Hershko et al., 1979) and received 2004 Nobel Prize in Chemistry for this remarkable discovery (Kresge et al., 2006). Since then, peptide-based post-translational modification of proteins has been shown to modulate a wide array of biological processes inside the eukaryotic cell. The post-translational modifiers SUMO (Small Ubiquitin-like Modifier) and Ub are the most important members of ubiquitin-like proteins. These are evolutionarily conserved from yeast to human and determine the fate of a plethora of cellular proteins (Welchman et al., 2005; Hochstrasser, 2009).

SUMO is best-characterized amongst the members of Ub-related proteins and the SUMO pathway is essential for the viability of the eukaryotic cell (Hayashi et al., 2002; Nacerddine et al., 2005; Welchman et al., 2005; Saracco et al., 2007). Despite high structural similarity (Fig 1-1), SUMO has only ~18% amino acid sequence homology with Ub and largely works independently of Ub (Bayer et al., 1998; Muller et al., 2001; Welchman et al., 2005). Furthermore,

different surface charge distribution of both cousins suggests their distinct roles (Melchior, 2000; Sloper-Mould et al., 2001; Dohmen, 2004; Gill, 2004).

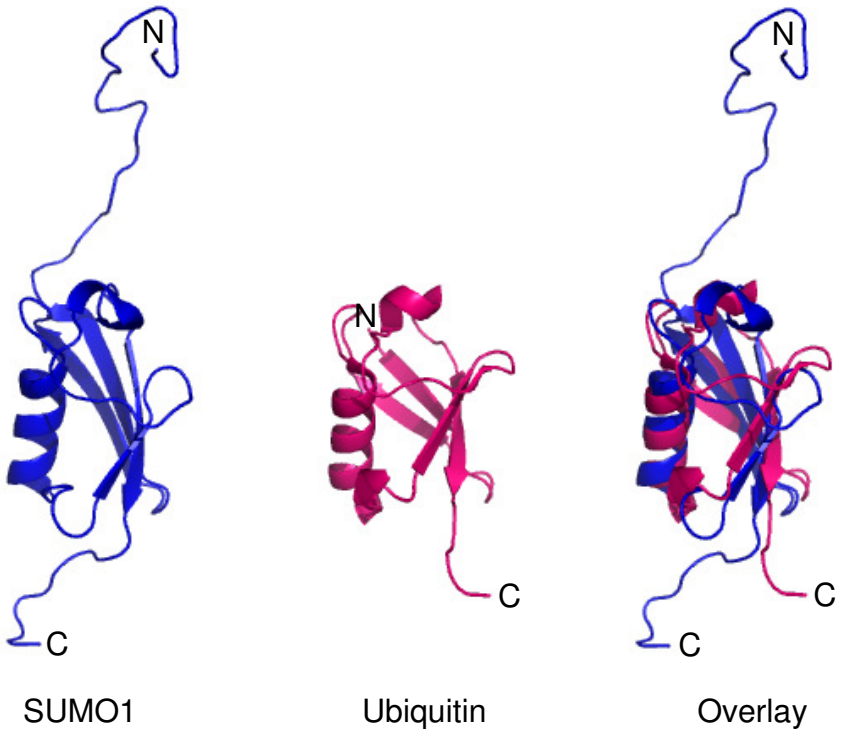

Figure 1-1 A ribbon representation of *Hs*SUMO1, ubiquitin and overlay of both. SUMOs show high structure similarity with ubiquitin. Graphics generated by using PyMol, DeLano Scientific LLC.

Apart from several non-degradative functions, Ub particularly forms poly-Ub chains on the proteins to be degraded by proteosomes (Finley, 2009) and so can SUMO, however, SUMO attachment does not lead the proteins directly to proteosome-mediated proteolysis like Ub does (Johnson and Gupta, 2001; Ulrich, 2009). Instead, SUMOylation modulates protein conformation, function, localization and stability and may even prevent proteins being ubiquinated and

subsequently degraded by the proteosome (Desterro et al., 1998; Hoege et al., 2002; Steinacher and Schar, 2005; Ulrich, 2005; Hofmann et al., 2009; Makhnevych et al., 2009). Recent studies, however, revealed some interesting findings that poly-SUMO chains of SUMO2 and 3 in mammals recruit Ub E3-ligases leading to ubiquitination and proteosome-mediated proteolysis (Ulrich, 2008; Geoffroy and Hay, 2009; Wang and Prelich, 2009). *Arabidopsis* SUMO1 and 2 (SUMO1/2) have also been shown to form poly-SUMO chains with yeast PCNA (proliferating cell nuclear antigen) *in vitro* but not SUMO3 (Colby et al., 2006). Both SUMO and Ub may attach to the same residue of a protein at different times dictating distinct protein function and localization (Hoege et al., 2002). In some cases, SUMO competes with ubiquitin for the same lysine (Lys) (Hoege et al., 2002), yet the question of preferred targets is still open. Interestingly, SUMO may itself be ubiquitinated resulting in SUMO-Ub chains (Tatham et al., 2008), or phosphorylated at an N-terminal serine (Matic et al., 2008). However, the biological relevance of these modifications is not very clear.

In historical perspective, the SUMO gene *Mif2* was first identified in yeast in 1995 encoding a centromeric protein (Meluh and Koshland, 1995) which was later designated as *SMT3*. One year later, SUMO was found covalently attached to a 70-kDa protein RanGAP1 (Ran-GTPase-activating protein 1) in the nuclei of rat liver cells and this conjugation was recorded as ATP dependent (Matunis et al., 1996; Mahajan et al., 1997). In plants SUMO was first reported in tomato and named T-SUMO (Hanania et al., 1999).

Soon after the discovery of SUMO, there was a burst of papers focusing on the SUMOylation pathway and its functional attributes. Initially, SUMO received several names in different organisms and reports such as sentrin, Ubl1, Gmp1, Pic1 and SMT3 (Johnson and Blobel, 1997; Melchior, 2000; Johnson, 2004) before a consensus was built. Broadly speaking, SUMOs are small globular eukaryotic proteins around 100 amino acids in length and 12-kD in mass having a *ββαββαβ* fold common to Ub-like proteins (Mayer et al., 1998; Melchior, 2000; Welchman et al., 2005). SUMO is synthesized as a pre-protein (SUMO precursor), and after being proteolytically cleaved through its C-terminus di-glycine motif, becomes covalently attached to the substrate proteins through an ATP dependent reaction cascade involving activation (E1s), conjugation (E2) and ligation (E3) enzymes (Kurepa et al., 2003; Schwartz and Hochstrasser, 2003; Johnson, 2004; Colby et al., 2006). The whole process is mechanistically analogous to ubiquitination but requires its own E1, E2 and E3 enzymes which work independently of the Ub pathway (Ulrich, 2009).

As a consequence, a protein conjugate is formed bearing the SUMO tag linked via an isopeptide bond between C-terminal glycine of SUMO and the ε-amino group of the lysine within the SUMO consensus motif ψ-**K**-X-D/E (where, ψ is a large hydrophobic residue, K is the target lysine, X could be any amino acid and D/E correspond to glutamate or aspartate in the substrate, respectively) (Johnson and Blobel, 1999; Minty et al., 2000; Rodriguez et al., 2001; Bernier-Villamor et al., 2002; Song et al., 2005; Ulrich, 2009). Nevertheless, SUMOylation at non-consensus motifs called SUMO interacting motifs (SIMs),

and non-covalent SUMO interactions with other proteins have also been observed (Denison et al., 2005; Zhu et al., 2008; Blomster et al., 2009). All SUMOs bear some additional C-terminal residues beyond the di-glycine motif (precursor SUMO), which are cleaved before SUMO becomes mature and ready to anchor its substrate. A family of SUMO specific proteases (ULPs) are capable of cleaving SUMOs off their targets making SUMOylation a highly dynamic, transient and reversible process (Chosed et al., 2006; Hay, 2007; Mukhopadhyay and Dasso, 2007).

1.2.1 The SUMOylation Machinery

Arabidopsis has eight potentially functional SUMO genes (*SUMO1-8*) which are similar to those in animals and fungi, while *SUMO9*, having a partial coding region, is most probably a pseudogene (Kurepa et al., 2003; Novatchkova et al., 2004; Miura et al., 2007a; Saracco et al., 2007) (Table 1-1). SUMO 1 and 2 (SUMO1/2), being the most important SUMOs in *Arabidopsis*, correspond to the human SUMO2 and 3 (SUMO2/3). SUMO1/2 have 97% amino acid sequence identity after maturation (Fig 1-2). The same is true for human SUMO2 and 3 (SUMO2/3). *Arabidopsis SUMO1/2* are functionally redundant as there is no clear phenotype if only one of them is knocked out (van den Burg et al., 2010). Importantly, double mutants of *SUMO1/2* and *SUMO2/3* are lethal both in plants and animals, respectively (Dohmen, 2004; Saracco et al., 2007; Zhao, 2007). *Arabidopsis SUMO4* and SUMO*6*, and *SUMO7* and *SUMO8* are relatively identical, while *SUMO3* and *SUMO5* are distinct (Fig 1-2, B and C). *SUMO1/2* are the highly expressed SUMOs in *Arabidopsis* followed by *SUMO3* and

SUMO5 and these are considered as the functional *SUMO* paralogues (Saracco et al., 2007; Budhiraja et al., 2009; van den Burg et al., 2010). Absence of a detectable transcript signal in reverse transcriptase (RT)-PCR and gel-blot analysis suggests *SUMO4* and *SUMO6*-8 either have a very low expression level or express only in certain tissues under specific circumstances (Kurepa et al., 2003; Saracco et al., 2007). Their role has not yet been described in any published report to our knowledge. Presence of a similar SUMO machinery has been recently published in rice and *Populus* (Chaikam and Karlson, 2010; Reed et al., 2010). In mammalian cells, four SUMO isoforms (SUMO1, 2, 3 and 4) have been reported. Mammalian SUMO1 shares roughly 50% sequence identity with SUMO2/3 (Saitoh and Hinchey, 2000) while SUMO4, being unable to form a covalent bond with substrate, has been found to have non-covalent interactions with other proteins, is mainly expressed in kidneys and lymph nodes and contributes in type I diabetes pathology (Guo et al., 2004; Owerbach et al., 2005; Wang and She, 2007). Mammalian SUMO2 and 3 also have an N-terminal ψ-K-X-D/E motif which allows them to form poly-SUMO chains (Tatham et al., 2001). A single SUMO gene is present in nematodes, yeast and insects which is essential for their viability except for fission yeast *Schizosaccharomyces pombe* where mutation in *pmt3* encoding SUMO renders severe developmental defects, yet the cells remain viable (Johnson and Blobel, 1997; Johnson et al., 1997; Huang et al., 1998; Tanaka et al., 1999; Long and Griffith, 2000; Jones et al., 2002).

Table 1-1 The SUMOylation machinery in *Arabidopsis*.

Gene	Locus	Length (aa)	MW (kDa)	References
SUMOs				
SUMO1	At4g26840	100	11	(Lois et al., 2003; Novatchkova et al., 2004; Colby et al., 2006; Miura et al., 2007a; Saracco et al., 2007; Lois, 2010; van den Burg et al., 2010)
SUMO2	At5g55160	103	12	
SUMO3	At5g55170	111	13	
SUMO4	At5g48710	114	13	
SUMO5	At2g32765	108	12	
SUMO6	At5g48700	117	14	
SUMO7	At5g55855	59	7	
SUMO8	At5g55856	97	11	
SUMO9	Pseudogene			
SUMO activating enzymes (E1)				
SAE1a	At4g24940	322	36.1	(Lois and Lima, 2005; Miura et al., 2007a; Saracco et al., 2007)
SAE1b	At5g50580	320	35.7	
SAE2	At2g21470	625	70	
SUMO conjugating enzymes (E2)				
SCE1	At3g57870	160	18	(Lois and Lima, 2005; Colby et al., 2006)
SUMO ligases (E3)				
SIZ1	At5g60410	873	96	(Miura et al., 2005; Catala et al., 2007; Miura and Hasegawa, 2008; Ishida et al., 2009; Miura and Hasegawa, 2009)
MMS21 / HPY2	At3g15150	249	28	
SUMO specific proteases (ULPs)				
ULP1a	At3g06910	502	58	(Kurepa et al., 2003; Novatchkova et al., 2005; Chosed et al., 2006; Colby et al., 2006; Miura et al., 2007a; Xu et al., 2007; Conti et al., 2008; Conti et al., 2009)
ULP1b	At4g00690	242	29	
ULP1c / OTS2	At1g10570	571	66	
ULP1d/OTS1	At1g60220	584	67	
ULP2a	At4g33620	783	89	
ULP2b	At1g09730	984	112	
ULP2c	At4g08430	808	93	
ULP2d	At4g04130	Transposons		
ULP2e	At4g19310			
ULP2f	At5g34990			
ULP2g	At2g05560			
ULP2h	At2g16180			
SENPlike1	At5g60190	226	26	
ESD4	At4g15880	489	56	

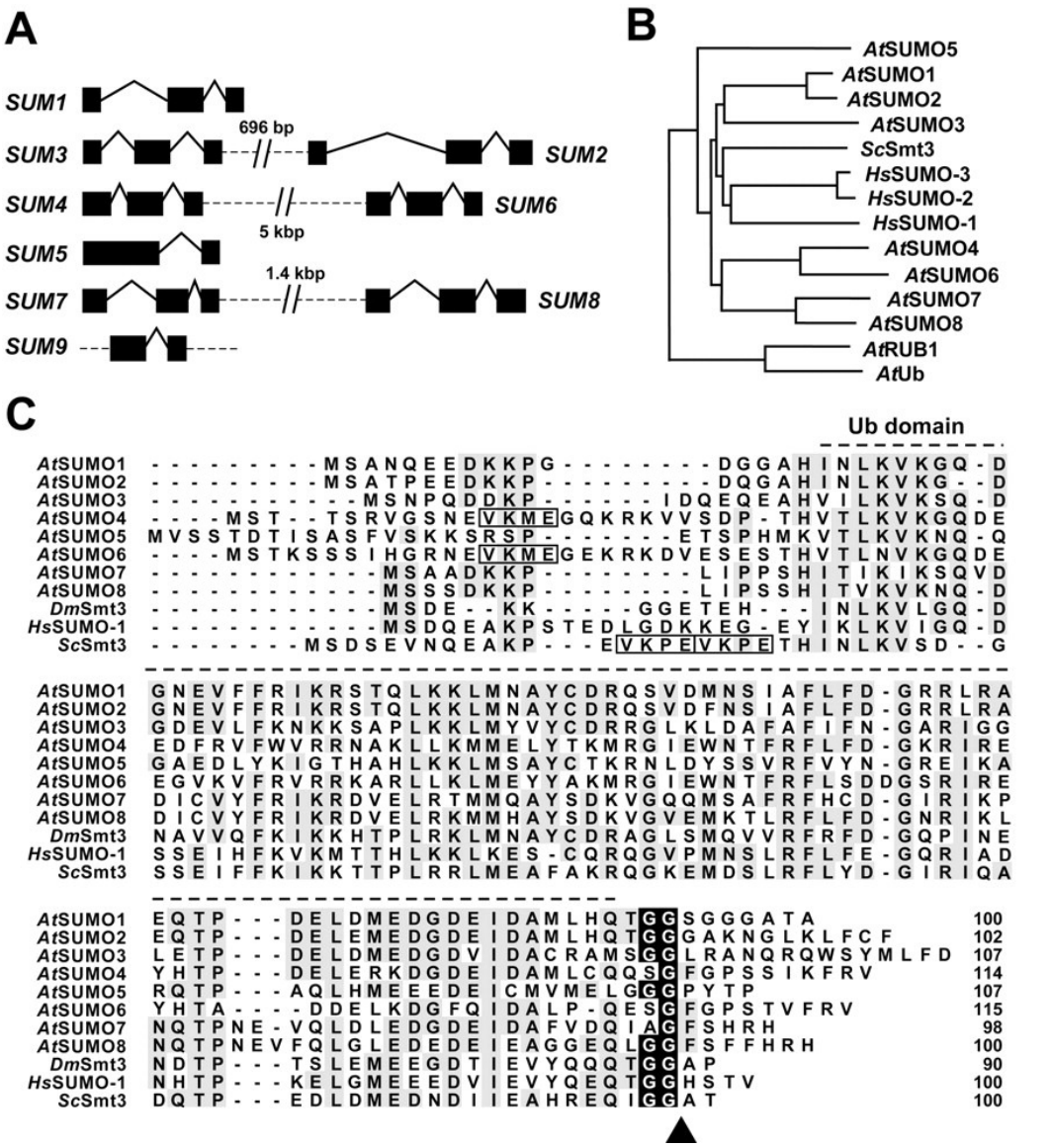

Figure 1-2 SUMO genes in *Arabidopsis.*

A) Black boxes and lines show exons and introns, respectively. Dotted lines separate two adjacent SUMOs by the length shown in base pairs (bp).

B) Phylogenic relationship between *Arabidopsis* (*At*), human (*Hs*) and yeast (*Sc*SMT3) SUMOs, Ubiquitin (Ub) and a plant Ub-like protein RUB1.

C) Amino acid sequence alignment of all components detailed in 'B'. Pre-SUMO is cleaved through di-GG to become active.

Source: Kurepa et al. (2003) J. Biol. Chem. 278(9): 6862–6872

A set of three SUMO activating enzymes (SAEs) i.e. SAE1a (also called Aos1 in mammals), SAE1b and SAE2 (also called Uba2 in mammals) (Table 1.1) make an E1 heterodimer, analogous to the Ub activating enzyme complex. The activation process recruits the mature SUMO in an ATP-dependent enzymatic reaction forming an E1-SUMO complex. Unlike Ub where ~37 ubiquitin-conjugating enzymes are present (Bachmair et al., 2001), there are two genes encoding E2 enzymes (SCE1a and SCE1b) in *Arabidopsis*. SCE1b is truncated due to the absence of the first 53 N-terminal residues and is non-functional (Kurepa et al., 2003). Hence, SUMO conjugation is carried out by a sole E2 enzyme SCE1a (generally annotated as SCE1 or SCE) which transfers SUMO to the substrate protein (Lois et al., 2003). This process of transfer may be mediated by SUMO E3-ligases. In *Arabidopsis*, for instance, SIZ1, MMS21/HPY2 are the only know SUMO E3 ligases to date (Miura et al., 2005; Catala et al., 2007; Ishida et al., 2009) (Table 1-1). Unlike ubiquitination, where ligation is strictly determined by E3-ligases which are regarded as essential, E3-ligases are not primarily essential for SUMOylation. Indeed, they determine the substrate specificity or help choose the SUMOylation site within the same substrate (Pfander et al., 2005; Bergink and Jentsch, 2009). In *Arabidopsis*, a family of nine SUMO-specific proteases also known as Ub-like proteases (ULPs) have been reported which are capable of deconjugating SUMO from its targets making SUMOylation a highly dynamic and reversible process (Table 1-1).

1.2.2 Mechanism of SUMOylation

In principle, SUMO proteins are produced in precursor form and are cleaved through their di-glycine motif close to the N-terminus by ULPs. The process called maturation transforms a precursor SUMO into a mature form (Fig 1-3). There are a few exceptions to this di-glycine cleavage e.g. *Arabidopsis* SUMO4, 6 and 7 do not actually have a di-glycine motif. Presumably, these SUMOs are cleaved through single glycines instead; nevertheless, there is no published data to support this assumption. The mature SUMO undergoes activation by SUMO activating enzymes (E1) which form a heterodimer comprising a smaller 36 kDa sub-unit of either SAE1a or SAE1b (SAE1a and SAE1b have 81% amino acid sequence identity and presumably work redundantly), and a single 70 kDa SAE2 (Desterro et al., 1999; Kurepa et al., 2003; Novatchkova et al., 2005). SAE2 has a catalytic Cys domain (159-386 amino acids) and a Ubl domain (442-549 amino acids) which are linked together via a zinc-finger motif in the protein tertiary structure (Lois and Lima, 2005). At first, the carboxyl group of the exposed glycine of SUMO gets adenylated by attacking an ATP molecule releasing a pyrophosphate. The adenylated SUMO makes a thioester bond with the catalytic Cys thiol of SAE2 releasing AMP and is bound between catalytic Cys and Ubl domains of SAE2 which is already a heterodimer with SAE1 (Walden et al., 2003; Lois and Lima, 2005; Olsen et al., 2010).

SUMO is then transferred to SCE1 (E2) forming a thioester bond with its active site Cys (Cys^{93} and Cys^{94} in animals and plants, respectively). Substrate

containing the ψ-K-X-D/E motif directly interacts with SCE1-SUMO complex, while none of the flanking residues are in direct contact with the SCE1 active site. This suggests that the tetrapeptide motif having nucleophile acceptor Lys is sufficient to catalyze the formation of an isopeptide bond between SUMO and the substrate (Bernier-Villamor et al., 2002; Tatham et al., 2003). In animals, Asp^{100} and Lys^{102} lying close to the active site Cys in protein tertiary structure are also considered important for substrate recognition (Tatham et al., 2003). Apart from *SUMO1/2* in *Arabidopsis*, genes encoding E1 and E2 are essential and loss-of-function mutations in *SAE2* and *SCE1* are lethal (Saracco et al., 2007).

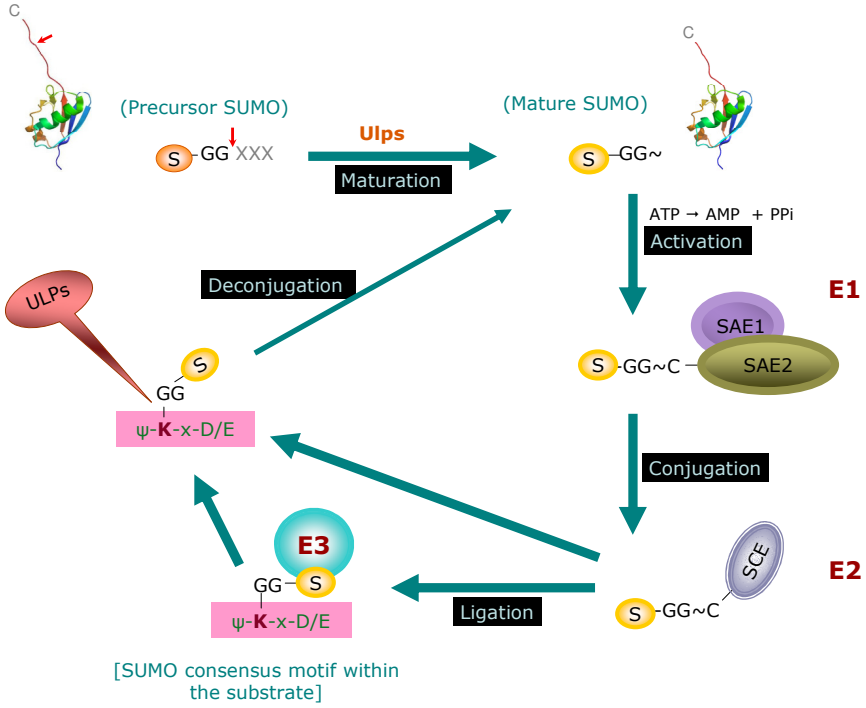

Figure 1-2 The SUMOylation cycle.

The precursor SUMO is cleaved through its di-glycine motif. After going through a series of enzymatic reactions viz. activation, conjugation and ligation, mature SUMO binds to its target at lysine. Being a reversible process, de-SUMOylation is carried out by SUMO-specific proteases. SUMOs can also form poly-SUMO chains with the substrate because of self-SUMOylation.

Unlike Ub where an E3-ligase is inevitable for ubiquitination, SUMOylation does not necessarily require an E3 either *in vivo* or *in vitro*. Though, E3s facilitate the transfer of SUMO by bringing the E2-SUMO complex and the substrate together making SUMOylation more efficient (Desterro et al., 1999; Johnson, 2004; Hay, 2005). The E3-ligases in plants have a characteristic SP-RING [Siz-PIAS (SAP (scaffold attachment factor protein) and Miz (MSX2-interacting zinc-finger) - protein inhibitor of activated STAT (signal transducer and activator of transcription)-RING] domain (Cheong et al., 2009; Cheong et al., 2010).

*At*SIZ1 is a homologue of mammalian PIAS-like proteins exhibiting E3-ligase activity and is associated with several cellular responses in plants (Kurepa et al., 2003; Miura et al., 2005; Lee et al., 2007; Miura et al., 2009; Miura et al., 2010) and cell division in yeast (Johnson and Gupta, 2001). A single PIAS-like protein in *Drosophila* is involved in chromosome organization and stability (Hari et al., 2001). These PIAS-like proteins do not covalently attach with either the E2-SUMO complex or substrate but provide a SUMO binding platform by folding around the E2, orientating the target lysine and stabilizing E2-target interaction by improving their affinity (Hochstrasser, 2001; Melchior et al., 2003; Johnson, 2004; Miura et al., 2005; Reverter and Lima, 2005). A large number of substrates have been identified in mammals targeted by the PIAS family of proteins which constitute most of the E3 ligases (Nishida and Yasuda, 2002; Schmidt and Muller, 2002; Melchior et al., 2003). Some non-PIAS mammalian E3-ligases devoid of a RING-finger like domain include RanBP2 present in nuclear pore complexes

(Pichler et al., 2002; Pichler et al., 2004) and Polycomb group (PcG) proteins which enhance SUMOylation of a transcriptional corepressor CtBP (C-terminal binding protein) (Kagey et al., 2003; Wotton and Merrill, 2007). Another novel protein RSUME (RWD-containing SUMOylation enhancer) was also found having SUMO E3-ligase activity by enhancing SUMOylation of an inhibitory mammalian protein IκB (Carbia-Nagashima et al., 2007).

A family of deSUMOylating enzymes known as ULPs (Ub-like proteases) in yeast and *Arabidopsis*, and SENPs (Sentrin-specific proteases) in mammals are capable of deconjugating SUMO or depolymerizing poly-SUMO chains from their substrate (Mukhopadhyay and Dasso, 2007; Kim and Baek, 2009), by cleaving the amide bond between SUMO and the substrate lysine. These isopeptidases indeed hold a dual role by also carrying out C-terminal hydrolysis through the di-glycine cleavage of precursor SUMOs turning them to mature forms (Melchior et al., 2003; Chosed et al., 2006).

Apart from six SUMO-specific proteases or so called SENPs in mammals (Hay, 2007; Lima and Reverter, 2008; Gill, 2010), two SUMO (Smt3)-specific proteases in yeast (Ulp1 and Ulp2) (Li and Hochstrasser, 1999, 2000; Mukhopadhyay and Dasso, 2007) and nine in *Arabidopsis* have been reported so far (Kurepa et al., 2003; Chosed et al., 2006; Miura et al., 2007a; Conti et al., 2008). ULPs have a catalytic triad Cys–His–Asn lying within the 200 amino acid long conserved catalytic domains (Melchior et al., 2003) while a localization signal may be present within the N terminal e.g. in the case of mammalian SENP1 (Bailey and O'Hare, 2004). ULPs are highly selective for each SUMO paralogue

and do not compensate for each other (Gong and Yeh, 2006; Hay, 2007). These specificity determinants are believed to lie within the SUMO paralogues next to the diglycine motifs (Xu and Au, 2005; Shen et al., 2006; Hay, 2007).

In two coinciding reports, the *Arabidopsis* ULP1a, ULP1c ULP1d and ESD4 were shown to hydrolyse both SUMO1 and 2 *in vitro* to their mature forms, and at the same time, exhibited isopeptidase activity for RanGap1-SUMO or *Sc*PCNA-SUMO conjugates *in vitro* (Chosed et al., 2006; Colby et al., 2006). Only ULP1a and a bacterial virulence factor XopD (*Xanthomonas* outer protein D) was found specific for SUMO3 processing and SUMO5 specific ULP has yet to be identified (Chosed et al., 2006; Colby et al., 2006). This intimates the presence of more SUMO specific proteases undertaking important functions.

1.2.3 Consequences of SUMOylation

1.2.3.1 Roles of SUMOylation in animals

The functional diversity of SUMOylation can be appraised by the fact that about 15% of human proteins are potential SUMO targets (Yang et al., 2006; Makhnevych et al., 2009) while hundreds have been experimentally confirmed (Zhou et al., 2004; Zhao, 2007; Budhiraja et al., 2009). SUMOylation is extensively studied in animals and its role has been associated with numerous biological functions (Makhnevych et al., 2009) including DNA replication and damage repair pathways (Aragon, 2005; Pfander et al., 2005; Branzei et al., 2006; Lee and O'Connell, 2006; Branzei et al., 2008; Bergink and Jentsch, 2009; Galanty et al., 2009), regulation of gene expression by altering transcriptional factors, co-activators or repressors (Ross et al., 2002; Gill, 2003; Muller et al.,

2004; Beg and Scheiffele, 2006; Bossis and Melchior, 2006; Lyst et al., 2006; Liu and Shuai, 2008), chromosome segregation and cell cycle control (Nacerddine et al., 2005; Pfander et al., 2005; Schwartz et al., 2007; Nie et al., 2009), chromatin remodelling and genome integrity (Shin et al., 2005; Moldovan et al., 2007; Prudden et al., 2007; Chavez et al., 2010), cytosol/nuclear trafficking (Stade et al., 2002; Rajan et al., 2005; Lewis et al., 2007) and various human diseases, including cancers, type I diabetes and neurodegenerative disorders (Bohren et al., 2004; Steffan et al., 2004; Li et al., 2005; Beg and Scheiffele, 2006; Kim et al., 2006; Dorval and Fraser, 2007; Anderson et al., 2009; Subramaniam et al., 2009). Moreover, SUMOs have been found modulating a wide array of signalling networks in animals including metabolism, transport, cytoskeleton, development and senescence (Pichler and Melchior, 2002; Panse et al., 2003; Gill, 2005; Yamaguchi et al., 2005; Bischof et al., 2006; Chen et al., 2006; Li et al., 2006; La Salle et al., 2008; Chavez et al., 2010).

1.2.3.2 Roles of SUMOylation in plants

The presence of double the number of genes encoding SUMO proteins in *Arabidopsis* compared to mammals highlights a more pronounced role of SUMO modification in plant biology but much is yet to be uncovered. Apart from a few reports, most of the published literature in plants emerged using reverse genetic approaches characterizing the T-DNA mutant lines of *Arabidopsis* for the respective SUMO machinery genes with a primary focus on their functional attributes. There is almost a complete reliance on the yeast and mammalian research regarding the structural, biochemical and mechanistic aspects of

SUMOylation which are supposedly similar in plants. The first report on plant SUMOylation was published in 1999 where a tomato-SUMO protein (T-SUMO) was found interacting with ethylene inducing xylanase (EIX) from the fungus *Trichoderma viridae* which causes rapid induction of defence response by ET biosynthesis in tomato (Hanania et al., 1999). The induction of ET production and cell death was suppressed when T-SUMO was over-expressed, while anti-sense lines displayed reverse effects suggesting T-SUMO negatively regulates defence response (Hanania et al., 1999). SUMOylation in plants has been primarily associated with stress responses, development and flowering. Some preliminary data also linked SUMOylation to chromatin remodelling, RNA processing and protein synthesis (Budhiraja et al., 2009; van den Burg and Takken, 2009).

Considering the entire *Arabidopsis* SUMOylation machinery, the best characterized gene to date is *SIZ1* which encodes one of the two reported SUMO E3-ligases in plants (Catala et al., 2007). Mutant *siz1* plants show enhanced inorganic phosphate starvation response (Miura et al., 2005), accumulate more SA than wild-type (Miura et al., 2005; Jin et al., 2007; Lee et al., 2007), are hypersensitive to high and low temperatures (Yoo et al., 2006) and have lesser drought tolerance (Catala et al., 2007). *SIZ1* is expressed in all plant tissues and impaired *SIZ1* function in *Arabidopsis* causes a dwarf phenotype through SA mediated cell division and expansion defects which was rescued by overexpressing *NahG* in *siz1* deficient plants (Miura et al., 2005; Cheong et al., 2009; Miura et al., 2010). Mutant *siz1* plants flower earlier due to high SA levels (Jin et al., 2007) and display enhanced resistance against bacterial pathogens (Lee

et al., 2007). *SIZ1* was also found to negatively regulate ABA signalling by facilitating SUMOylation of ABA signal transducer ABI5 (abscisic acid insensitive 5) at K^{391} (Miura et al., 2009). *SIZ1* also modulates SUMOylation of MYC-like transcription factor ICE1 (inducer of *CBF/DREB1* expression 1) during cold stress signalling (Catala et al., 2007; Miura et al., 2007a; Miura et al., 2007b). The second plant E3 ligase *AtMMS21*, a human and yeast homologue like *SIZ1* (Potts and Yu, 2005; Branzei et al., 2006), was reported relatively recently. Mutant *atmms21* plants displayed defective root development caused by disrupted cell division and cytokinin signalling (Huang et al., 2009).

Apart from biotic stresses discussed in more detail in the following section, the role of SUMOylation has also been associated with several abiotic stress cues, especially, ABA signalling (Kurepa et al., 2003; Chaikam and Karlson, 2010). Increase in SUMO1/2 protein conjugates was reported in *Arabidopsis* plants exposed to high temperatures (Kurepa et al., 2003). In three independent experiments, *Arabidopsis* seedlings exposed to 5 mM H_2O_2, 30 mg/l canavanine and 7% ethanol showed increased SUMO1/2 conjugation 30 min after treatment but not SUMO3 (Kurepa et al., 2003) suggesting SUMO1/2 may be more important during stress responses compared to SUMO3. Using artificial miRNA (microRNA) gene silencing to knockdown *SUMO1/2* expression, it was shown that SUMO1/2 are essential for plant development, flowering and SA dependent responses, especially defence (van den Burg et al., 2010).

SUMOylation negatively regulates ABA signalling (Lois et al., 2003). Plants overexpressing *SUMO1/2* displayed an attenuated ABA induced root

growth inhibition and also led to the induction of stress responsive genes *RD29A* and *AtPLC1*, indicating important roles of SUMOylation in ABA mediated response (Lois et al., 2003). SUMO protease-deficient double mutants *ots1* and *ots2* (overly tolerant to salt 1 and 2; also known as *ulp1d* and *ulp1c*, respectively) showed increased sensitivity to high-salt stress induced by 100 mM NaCl which was partially rescued in 35S::*OTS1* over-expressing transgenic lines (Conti et al., 2008). Increased accumulation of SUMO conjugates after NaCl treatment was also significant in *ots1* and *ots2* single mutants (Conti et al., 2008) highlighting the importance of SUMO deconjugation carried out by these proteases during salt-stress response.

SUMOylation is also regarded as a key player in the phytochrome A signal transduction cascade (Ballesteros et al., 2001) and flowering time regulation (Reeves et al., 2002; Murtas et al., 2003; Jin et al., 2007; Conti et al., 2008). Mutation in *Arabidopsis ESD4* (a SUMO protease) results in extremely early flowering in short-day conditions (Reeves et al., 2002). Likewise, *ots1 ots2* double mutants exhibit early flowering both in short and long day conditions without displaying any floral or developmental defects suggesting *OTS1* and *OTS2* have a synergistic role (Conti et al., 2008; Conti et al., 2009). A similar situation was found in *eds4* mutant plants where plant height, shape of siliques and positioning of cauline leaves in the shoot was affected indicating a role of SUMO proteases in plant development (Kim and Baek, 2009).

1.2.3.3 SUMOylation and plant defence

Despite a wealth of information on SUMOylation in mammalian pathogenesis, the link between SUMOylation and plant disease resistance is not well explored. Long after the discovery of T-SUMO interaction with EIX from the fungus *T. viridae* and its involvement in defence induction (Hanania et al., 1999), increased resistance of *siz1* plants against bacterial pathogens was reported (Lee et al., 2007). Compared to wild-type, *siz1* plants were found more resistant against *Pst*DC3000 expressing *avrRps4* and not *avrRpm1* suggesting SIZ1 suppresses *EDS1/PAD4*-dependent SA accumulation and signalling through the TIR-NBS-LRR class of R proteins by facilitating SUMOylation of certain unknown targets (Lee et al., 2007). Genetic analysis revealed that *SIZ1* acts epistatically with *PAD4* and functions upstream of *PAD4* and SA. Furthermore, elevated *SID2* and *PR1, PR2*, *PR5* gene expressions along with constitutive expression of SAR in the absence of pathogens was also evident in *siz1* plants (Lee et al., 2007) indicating *SIZ1* down-regulates defence signalling.

Knocking down *SUMO1/2* expression by amiRNA triggered gene silencing showed that SUMO1/2 are critical for effective defence response (van den Burg et al., 2010). To our surprise, knockdown *sumo1/2* lines as well as constitutively overexpressing *SUMO1, 2* and *3* plants displayed elevated *PR1* gene expression, accumulated more SA and SAG (SA-2-O-β-D glucoside) and displayed enhanced basal resistance against *Pst*DC3000. On the other hand, these knockdown mutant lines and overexpressors showed delayed HR after *Pst*DC3000 challenge expressing *avrRpm1* suggesting a tight regulation of SUMO

expression is required for normal defence response (van den Burg et al., 2010). Furthermore, SUMO3 expression was induced by exogenous SA application or peptide FLG22 infiltration in *Arabidopsis* leaves. It was proposed that SUMO3 acts downstream of SA while SUMO1 and 2 fine-tune defence activation upstream of SA (van den Burg et al., 2010)

Interaction between tobacco SCE1 (*Nb*SCE1) and geminivirus replication proteins Rep/RepAC1 has also been reported in yeast-two-hybrid assays suggesting an important role of SUMOylation in viral DNA replication during geminivirus infection (Castillo et al., 2004). Similarly, SUMOylation of a vaccinia virus protein A40R is essential for its nuclear localization to assist the delivery of the viral genome to the replication site (Schramm and Locker, 2005). Interestingly, the viral proteins acquiring SUMOs in animals are encoded by DNA viruses which replicate in the nucleus and SUMOylation indeed disrupts their nuclear localization (Schramm and Locker, 2005; Boggio and Chiocca, 2006).

Pathogens infect and colonize plant and animal hosts with very similar strategies and the R proteins in diverse hosts share several common features. Therefore, both plants and animals combat pathogen intrusions in very similar ways (Staskawicz et al., 2001). There is enough evidence to support the hypothesis that SUMO proteins facilitate infections by certain pathogens. Certain pathogen proteins need to be SUMOylated or these virulence factors deSUMOylate proteins in the host in order to exert their functions. A bacterial type-III effector YopJ (*Yersinia* outer protein J), which acts as a SUMO1-specific Cys protease both in animals and plants, blocks MAPK signalling and activation

of a cell death controlling transcriptional factor NF-κB (nuclear factor Kappa B) pathway by disrupting SUMOylation. This eventually limits immune activation in animals and hypersensitive cell death in tobacco (Orth et al., 2000; Orth, 2002). Some succeeding reports also suggested that YopJ acts as a deubiquitinase in NF-κB signalling (Zhou et al., 2005) or acts as an acetyl-transferase which blocks phosphorylation of MAPKK6 inactivating the immune pathway (Mukherjee et al., 2006). It is likely that YopJ has all these activities under different circumstances to mimic host defences. A human pathogenic bacteria *Listeria monocytogenes* has recently been shown to cause deSUMOylation of SUMO-conjugates and proteosome-independent degradation of Ubc9 triggered by its virulence factor listeriolysin O (LLO) to facilitate infection (Ribet et al., 2010). Overexpressing *SUMO1* or *SUMO2* in HeLa cells significantly reduced bacterial growth measured 7 h post-infection which indicates that increased SUMOylation renders more resistance in mammals (Ribet et al., 2010). Taken together, these findings indicate that virulent pathogens tend to bring down the cellular SUMO-conjugates levels to become successful.

Similarly, the bacterial effector XopD (*Xanthomonas* outer protein D from *Xanthomonas campestris*) is a plant specific SUMO protease which acts in the cells' nuclei and suppresses host defence and cell death by inhibiting SA and JA induced transcription and activation of defence genes by protein deSUMOylation (Hotson et al., 2003; Chosed et al., 2007; Kim et al., 2008; Kay and Bonas, 2009). Moreover, *Xanthomonas* YopJ (*Yersinia* outer-protein J) -like effector AvrXv4 was reported to have SUMO isopeptidase activity in the cytoplasm during

infection (Roden et al., 2004) suggesting deSUMOylation is an important strategy to aid pathogenicity (Innes, 2003).

Chapter 2

2 Materials and Methods

Unless otherwise stated, all chemicals and primers were purchased from Sigma (Sigma-Aldrich, UK) and all the restriction and ligation enzymes from New England Biolabs (NEB, USA). Water means autoclaved and deionized distilled water. The labwares, buffers and reagents were autoclaved before use where necessary.

2.1 SUMO T-DNA Insertion Lines

Arabidopsis thaliana (*Arabidopsis*) genes encoding five SUMO proteins (*SUMO1-5*), E1 enzymes (*SAE1a*, *SAE1b*, *SAE2*), E2 enzyme (*SCE1*) and SUMO proteases (*ULP1a*, *ULP1b*, *ULP1c* and *ESD4*) were identified in The Arabidopsis Resource Database (TAIR) (www.arabidopsis.org). The T-DNA insertion mutants in *Arabidopsis* ecotype Columbia (Col-0) background were further investigated for the availability of the mutant lines in Nottingham Arabidopsis Stock Centre (NASC), GABI-Kat (Genomanalyse im Biologischen System Planze) or SAIL (Syngenta Arabidopsis Insertion Lines) collections and seeds were ordered (Table 2-1). In most cases, different mutant alleles available were ordered for the same gene with different T-DNA insertions in exon, intron and promoter regions. Seeds for each mutant strain were received as a segregating generation for the T-DNA insertion.

Table 2-1 The T-DNA insertion mutants and primers used for genotyping.

Loci	Gene	Accession No./Insertion			Insertion-specific primer pairs
At4g26840	*SUMO1*	GABI_658E02	Intron	L	TCATGGAGAGTGGTGGATAGG
				R	AATCACATGGCTCTCTTTTGG
		GABI_675B02	Intron	L	TTAAGAAGGGGTTCAGGTTCAG
				R	TTGGCTGATGTTTGTGCTTC
At5g55160	*SUMO2*	SALK_129775	Exon	L	TTGGGATCCAAGGAACCGTGG
				R	ACTGTGACCGTCAGTCTGTGG
At5g55170	*SUMO3*	SALK_123673	Exon	L	GCGGGATTGCAGAACTTGAGG
				R	GATGAGTGGTGGTCTACGAGC
		SALK_083859	Promoter	L	TACGTCGACCGTCATAGTGTG
				R	CTAAATGGTCCATCTCCGTCC
At5g48710	*SUMO4*	SALK_132447	Exon	L	GTTGCTTCCCTTTCCTCAAAG
				R	TTGTCCCCTTCGTACTGTCAC
At2g32765	*SUMO5*	GABI_606E01	Exon	L	CGAATTAACAACAACGTTAAGAGAG
				R	AGACCGACGATAAACGAAAGC
		SAIL_504_B06	Exon	L	GAGACTTAAGATGCAACATTGAAAC
				R	TTGGAGAGACCGACGATAAAC
		SALK_119313	Promoter	L	TCTTCTTCTTCCATGTGCAGC
				R	ATGACGATGATGATGATGACG
		SAIL_770_G01	Exon	L	CTCTGAACCCAGAAGAAACCTC
				R	GTTAAGATTCGGGTCGAGTGG
		SALK_085812	Promoter	L	AGCCTAATCTGTTTATCCCGC
				R	TGTGATGTTTTTGGTTTGGAG
		GABI_370D01	Promoter	L	TCTTCTTCTTCCATGTGCAGC
				R	ATGACGATGATGATGATGACG
At4g24940	*SAE1a*	SALK_060834	intron	L	CTTGAAGAGATGGTGGAATGCG
				R	CTGCTTTGATCACCTCCTGCC
		SALK_007598	5′ UTR	L	GCCACTAGAAAAATTACCACCG
				R	TGAAAAGAACAAGGGGATGAG
		SALK_044105	Promoter	L	TACAAATTCCATAATGTCCCG
				R	CAACTCTCTCACAGTGTTTGCC
At5g50580	*SAE1b*	No mutant available			
At2g21470	*SAE2*	SALK_053023	Exon	L	CTCATGTTGGGCAGTCTAAGGC
				R	AGCCGCTTCAATGTTAGAACC
		SALK_ 020005	Exon	L	CAAACCAAACGGTGTGAAGAG
				R	TCTTCCATTTCACTGAATCACG
		SALK_026931	Promoter	L	CCAATGGGCATGATAAAACAC
				R	GACTTCAATTTGAAAACACCCC
		SALK_094819	Promoter	L	CTCCAACCATAAGCACTTTCG
				R	CCAATGGGCATGATAAAACAC
		SALK_014443	Promoter	L	CTCCAACCATAAGCACTTTCG
				R	CCAATGGGCATGATAAAACAC
		SALK_020830	Exon	L	TCCTTCTCCCATTCTTAACGG
				R	AAATCTTTTCATAATCAACCGATTC
At3g57870	*SCE1*	SALK_071596	Intron	L	TTTCCGACCATTCTGTTTGAC
				R	AAGGGAAGAGACTGGTGAAGC
At5g60410	*SIZ1*	SALK_034008	Exon	L	CCTAGCCTAAGCGAGATCCAG
				R	AAAGAGAGAGTGAGCGAAGGG

		SALK_023805	Exon	L	CAATGAGTAGTAGTGCTACTGG
				R	GAGATCCAGTCTTCACTACG
		SALK_065397	Exon	L	GAGCTGAAGCATCTGGTTTTG
				R	CACGACAGATGAAGCATTGTG
At4g00690	*UPL1b*	SALK_110379	Exon	L	TTTCAGCTCGTAAGCGGCTCC
				R	GCTCGCAGTCTCAGTATCTCC
At1g10570	*ULP1c*	SALK_151423	Exon	L	TTTGTTTTGCATGTTTCCTAAAAC
				R	CAGAATGATGCAATTGCAGAG
		SALK_067439	Exon	L	CTAGGTACGTGCAACATCACG
				R	TCGTTGATCATATTGGGAAGG
		SALK_050441	Exon	L	AGGAGGATCAGTGGTTGAGTG
				R	GACATACCTTCCTGCAGCTTG
At1g60220	*ULP1d*	SALK_029340	Intron	L	TTCAGATGTTTTACCGCAAGG
				R	TCAAGTAGGAAGTTTTCCTGGC
		SALK_022798	Exon	L	GATGATGCAAGGAGGCTAGTG
				R	CCCATGATTCAAGGAACTTTG
At4g15880	*ESD4*	SALK_142934	Exon	L	GGAATAAAAGGTTCACGAGGC
				R	AACCCTTTACGAAATTCACCG
		SALK_032317	Intron	L	TTCATGGGATACAGAAGCCAG
				R	CTTATGCAAAGTGCGGAGAAG
		SALK_113130	Promoter	L	TCGTAAAGGGTTGGTTGATTG
				R	TTTGTAGAGGTTTTACTGTAAAACATG
		SALK_083174	Exon	L	AACCAAAAGCCACTTCAAAAAC
				R	CAACTTCCTCCTTTTCAACCTC
		SALK_083180	Exon	L	AAATTGTAAACATATGGTGGTGTTG
				R	ACACCAGAAGAAAACGAAGGC
		SALK_123045	Exon	L	GCAGTCTCATAGGCGTCTTTG
				R	AAGAAATTTGCGTCCTTTTCG

2.2 Plant Growth Conditions

The plants were grown in pots filled with soil comprising peat moss, vermiculite and sand (4:1:1) at 22 °C, 65% relative humidity and 16 hrs photoperiod in the environmentally controlled growth room under the illumination of 70-100 µmol m^{-2} sec^{-1} emitted by general electrical florescent tube-lights. The seeds were stratified in the dark at 4 °C for two-days to accomplish uniform germination before they were transferred to the growth-room. Ten-day old seedlings were transplanted in the pots containing soil. Twelve individual plants were selected at random to identify homozygous lines for T-DNA insertion. The

plants were individually labelled and the inflorescence was covered with perforated polythene bags one-week after bolting to prevent seed contamination and facilitate seed collection.

For aseptic growth, the seeds were germinated in the same growth conditions on petri plates having sterile ½ MS (Murashige and Skoog, 1962) salts and vitamins, 1% (w/v) sucrose, 0.4% (w/v) phytoagar. The pH was adjusted to 5.8 prior to autoclaving.

2.3 Genotyping for T-DNA Insertion

Genomic DNA (gDNA) extractions were performed by CTAB (Hexadecyl trimethyl-ammonium bromide) extraction buffer (100 mM Tris HCl, pH 8, 1.4 M NaCl, 20 mM EDTA, 2% CTAB and 0.2% β-mercaptoethanol) as described by Doyle and Doyle (1990). Genotyping was carried out by Polymerase Chain Reaction (PCR) in order to verify the presence of T-DNA inserts and identify homozygous T-DNA lines for the respective genes. Primers were designed by the SIGnAL (Salk Institute Genomic Analysis Laboratory) T-DNA Verification Primer Design online tool (http://signal.salk.edu/tdnaprimers.2.html). Two PCRs were carried out for each line to verify T-DNA inserts by using gDNA. Gene-specific primer pair was used in the first PCR (Table 2.1); while an additional T-DNA specific left-border primer was used in the second reaction depending on the T-DNA inserts from different sources, namely the SALK, SAIL or GABI-Kat collections (Table 2-2).

Table 2-2 The T-DNA left border primers used for genotyping SALK, SAIL or GABI-Kat lines.

Line source	Primer name	Primer sequence
SALK	LBb1	GCGTGGACCGCTTGCTGCAACT
SALK	LBa1	TGGTTCACGTAGTGGGCCATCG
SAIL	LB1	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
GABI -Kat	LB-GK	ATATTGACCATCATACTCATTGC

The gene-specific primers confirmed the presence of any wild-type gene copy on either chromosome, while the T-DNA specific primer in combination with a left-border primer confirmed the presence of a T-DNA insertion on homologous chromosomes interrupting the wild-type gene. Two gene-specific primers and a T-DNA left-border primer yielded a single band of ~ 0.5 kb in case of homozygous, two bands one ~ 0.5 kb and the other ~ 1 kb for a heterozygous, and only a single ~ 1 kb band for a wild-type plant with no T-DNA insertion at all. Seeds were collected from the lines identified as homozygous for T-DNA insertion and stored at room temperature in small butter paper bags.

2.4 Total RNA Extraction

Total RNA was extracted from the leaves of 3-4 week old plants using TRIzol® reagent. The leaf tissues (100 mg/plant) were ground in liquid nitrogen (LN_2) and were immediately transferred to pre-chilled 1.5 ml eppendorf tubes followed by the addition of 1ml TRIzol® reagent. Samples were vortexed vigorously before they were centrifuged for 10 min at 12,000*g* in the cold-room.

The supernatant was decanted into the fresh eppendorf tubes having 200 µl chloroform, mixed thoroughly and centrifuged again at 12,000*g* for 15 min at 4 °C. About 300 µl of aqueous phase was transferred to new tubes and 300 µl of each of isopropanol and NaCl/Na-Citrate salt solution (1.2 M NaCl and 0.8 M Na-citrate) was added and gently mixed to precipitate the RNA. The samples were allowed to sit at room temperature for 10 min followed by centrifugation at 10,000*g* for 10 min at 4 °C. The supernatant was discarded and the pellet was washed with 75% ethanol in DEPC (diethylpyrocarbonate)-treated water, air dried and resuspended in 50 µl DEPC-treated water. RNA concentration was measured by spectrophotometric absorbance at OD_{260} using thin quartz cuvettes and was calculated as $OD_{260} \times 40 \times$ (dilution factor).

2.5 Northern Hybridization

The probes were generated by PCR based amplification of desired fragments from *Arabidopsis* gDNA using specific primer pairs (Table 2-3). The fragments were purified after gel-electrophoresis using a gel extraction kit (Qiagen). RNA samples (10 µg each) were vacuum dried to reduce the total volume to ~8 µl before they were mixed with the cocktail (5.5 µl formaldehyde, 15 µl formamide and 1.5 µl of 10X MoPS buffer). The resulting volume was 30 µl for each sample. The RNA samples were denatured at 65 °C and separated by gel-electrophoresis on formaldehyde-agarose gel as described elsewhere (Sambrook and Russell, 2001).

Table 2-3 Primers used to generate DNA probes for northern hybridization.

Gene	Primers		Probe size (bp)
SAE2	F	TATGGGCAAAAGACCTGCTC	433
	R	TTGGCGAAGAACAACTTCAA	
ULP1b	F	CCTTCTTTTACGTTAAGGTTTGC	1000
	R	GGGGGCACTATTAGTCAGCTC	
ULP1c	F	TGAAGAGACAAAGAGCAATCG	328
	R	GTCCGTTGTTGGAACATCCT	
ULP1d	F	TCAGTCAAAGAAAGCCTTTGG	301
	R	AGTTTCATGGCCACTGGTGT	

A capillary blot was set up to transfer the samples onto Hybond™ -N hybridization membranes (GE Healthcare, UK) according to manufacturer's instructions. The transfer was validated by staining the membranes with methylene blue solution (0.3 M sodium acetate pH 5.5 and 0.03% (w/v) methylene blue) and the blots were de-stained with 1X SSC and 1% (w/v) SDS solution. The probes were labelled with radioactive ^{32}P-dCTP by using Prime-a-Gene® labelling kit (Promega) and hybridization was performed with Rapid-hyb™ buffer (Amersham Biosciences) according to the supplier's instructions. The membranes were washed 3-5 times with SSC buffer and exposed to X-Omat-AR™ imaging film (Kodak, USA) for 1-2 days at −80 °C and autoradiograms were developed in an X-ray developer.

2.6 Reverse Transcriptase (RT)-PCR

RNA extraction and quantification was carried out as before. To remove genomic DNA, RNA samples were treated with 1U of RNase-free DNase (Promega) per 1 µg RNA and incubated at 37 °C for 30 min in the supplied buffer. The reaction was stopped by adding stop-buffer and heat inactivated at 65

°C for 10 min. Reverse transcriptase (RT)-PCR was carried out by using Omniscript RT-PCR kit (Qiagen) according to the manufacturer's instructions. For cDNA synthesis, 1 µg RNA sample was denatured at 65 °C for 5 min and RT-reaction was performed at 37 °C for one hour in 1X reaction buffer, 0.5 mM dNTPs, 2.5 µM oligo(dt) primer, 4U of RNAse inhibitor and 2U of Omniscript RT in a total reaction volume of 10 µl. The reaction was heat inactivated and the cDNA was diluted 10-fold in DEPC-treated water. A normal 25-cycle PCR reaction was run in a thermal cycler using 5 µl cDNA per sample with forward and reverse primers listed (Table 2-4). Because of high gene sequence identity of *SUMO1* and *SUMO2*, the primers were designed by using 5′ UTR regions. The control reactions were run by using primers for *Actin1*. The products were separated on agarose gels, visualised under UV-transilluminator and images were taken.

Table 2-4 Primers used for RT-PCR analyses of SUMO genes.

Gene	Gene region		Primer sequence	Probe size (bp)
SUMO1	3′ UTR	F	CACTTCGTCCAGGTTTAGGG	272
		R	GCTTTTTACCGTTACCATACCAA	
SUMO2	3′ UTR	F	GCGGTTATTTTAATGGTTTTCC	181
		R	AAAATCCATAAACAAGCCCATT	
SUMO3	5′ UTR	F	TTTTCGATCATAATGGGCTTT	180
		R	AAAAATCCGTAACCAGATACCA	
SUMO5	5′ UTR + exon	F	TCCCATCATCTCTTTCTCACC	351
		R	GCAGGAGTCTGTCGAGCTTT	
Actin1	Exon	F	CATCAGGAAGGACTTGTACGG	257
		R	GATGGACCTGACTCGTCATAC	

2.7 Infection Experiments

2.7.1 Pathogen growth and inoculation

The virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*DC3000) and its avirulent strains carrying either *avrB* or *Rps4* were grown on King's broth (KB) liquid media (King et al., 1954), supplemented with 5 mM $MgSO_4$ and 50 mg/l rifampicin for virulent strain *Pst*DC3000, and an additional 50 mg/l kanamycin for both avirulent strains i.e. *Pst*DC3000(*avrB*) or *Pst*DC3000(*avrRps4*). The cultures were grown at 30 °C overnight. The following day, the cells were harvested by centrifugation at 4000*g* for 10 min and resuspended in 10 mM $MgSO_4$ solution. The OD_{600} was measured and the cultures were diluted in 10 mM $MgSO_4$ to the desired densities of colony forming units (cfu) before they were used as inoculum. An OD_{600} of 0.02 which corresponds to ~10^7 colony forming units (cfu)/ml was used for avirulent strains to monitor HR and changes in SUMO-conjugate levels. A relatively lower bacterial titre ~10^5 cfu/ml equivalent to OD_{600} 0.0002 was used to score disease symptoms and subsequent colony counts. While ~10^6 cfu/ml equivalent to OD_{600} 0.002 was used to monitor the changes in SUMOylation level after pathogen challenge. Inoculations were carried out by syringe infiltrating the abaxial side of the leaves of four-week old plants.

2.7.2 Electrolytic leakage assay for HR

Hypersensitive response (HR) involves localised cell death normally visible few hours after pathogen infection and lasts upto 24 – 48 hrs depending upon the inoculum density. To measure the cell death, three leaves per plant were

infiltrated with *Pst*DC3000(*avr*B) or (*avrRps4*). Each plant was counted as one treatment and each experiment was replicated at least four-times. The mock treatment was 10 mM $MgSO_4$. Infiltrated leaf tissues were harvested from each plant and nine leaf-discs (1 cm^2) per treatment were placed in petri-dishes containing 10 ml deionised distilled water to measure the electrolytic leakage as a consequence of HR and cell death. Data were recorded at different time points and expressed as electrolyte leakage at hours post infiltration (hpi). Eventually, all the leaf samples were boiled for 30 min, final conductivity readings were taken and the ion leakage was expressed as percentages of the total soluble salts (TSS) present in the leaf tissues.

2.7.3 Trypan blue (TB) staining for HR

To measure the cell death after HR, the leaves were stained with TB as described by Yun et al. (2003). The leaves were boiled in trypan blue solution (2.5 mg/ml TB, 25% lactic acid, 23% saturated phenol, 25% glycerol and 24% water) for 5 min and allowed to cool down. The stained leaves were rinsed with water and destained by dipping in saturated chloral hydrate solution (2.5 g/ml of water) for 24 hrs. The leaf samples were washed with water and mounted onto microscope slides with a drop of 70% glycerol. The slides were examined for cell death macroscopically and microscopically using a Lecia Wild M3C microscope and photographs taken.

2.7.4 Bacterial growth measurement

Infected leaves were collected 4 dpi after *Pst*DC3000 challenge. These leaves (100 ± 2 mg/plant) were ground in 2 ml of 10 mM $MgSO_4$ using a pestle

and mortar in three replications. Serial dilutions of $1/10^3$ and $1/10^4$ were made in water and 100 µl of final dilution was plated on NYG media having 50 mg/l rifampicin. Plates were incubated for 2 days at 30 °C and the number of colony forming units (cfu) was counted.

2.8 Protein Assays

2.8.1 Leaf protein extraction

Four-week old plants were challenged with *Pst*DC3000 as described. Leaf samples (200 ± 5 mg/plant) were collected at different time points (0, 6, 12, 24, 48 hpi) in three replications. The infected leaves were ground in liquid nitrogen and suspended in 3 volume of protein extraction buffer (50 mM Tris pH 7.9, 150 mM NaCl, 0.2% Triton X-100, supplemented with protease inhibitors; 2 mM N-ethylmaleimide, 10 mM iodoacetamide, 1 mM phenylmethylsulphonyl fluoride, 1 ug/ml pepstatine A and 1 ug/ml leupeptin). The samples were vortexed vigorously followed by incubation at 4 °C on a tilting table for one hour. The samples were then centrifuged at 17,000*g* for 30 min at 4 °C. The supernatant was transferred to eppendorf tubes and mixed. Protein concentrations were measured by Bradford assay (Bradford, 1971) using BSA (bovine serum albumin) as standard.

2.8.2 Plasmid constructs for recombinant protein expression

The cDNA was synthesized by using reverse transcriptase (RT)-PCR from *Arabidopsis* total RNA using first strand cDNA synthesis kit (Fermentas) according to the manufacturer's instructions. Full-length coding sequences for *SAE1a*, *SAE1b* and *SAE2* were PCR amplified using Phusion™ high-fidelity DNA polymerase (Finnzymes) with the primers given and restriction sites

underlined (Table 2-5). *SphI* and *BglII* restriction sites were generated by PCR amplification of cDNA, the PCR products were separated on agarose gel and the fragments were gel eluted. The PCR fragments were sub-cloned into pGEM-T easy vector and sequenced. The valid constructs were digested with *SphI* and *BglII* restriction enzymes, gel eluted and ligated in pQE70 expression vector (Qiagen). The ATG start codon was merged in *SphI* restriction site which resulted in an altered N-terminal 2nd amino acid as featured in pQE70 expression system.

Table 2-5 Primers used to clone SUMO enzymes in pQE70 expression vector.

Primer name	Primer sequence	Product size
SAE1a-SphI (F)	ACATGCATGCACGGAGAAGAGCTTACCGAGC	968+19 = 987 bp
SAE1a-BglII (R)	GAAGATCTAGAGGTAAAAGAGTCGGAAATG	
SAE1b-SphI (F)	ACATGCATGCACGGAGATGAGCTCACCGAGCA	956+19 = 975 bp
SAE1b-BglII (R)	GAAGATCTAAGCTTGTGGGATAGGTCCTC	
SAE2-Sph1 (F)	ACATGCATGCCTACGCAACAACAGCAATCCG	1878+19 = 1897 bp
SAE2-BglII (R)	GAAGATCTTTCAACTCTTATCTTCTTTTTGCTCACC	

The resulting plasmids pQE70-SAE1a, pQE70-SAE1b and pQE70-SAE2 were verified by sequencing and used for respective recombinant protein overexpression in the *E. coli* strain M15[pRep4] obtained from Qiagen, UK.

2.8.3 Site-directed mutagenesis

Mutations in Cys residues of SCE1 were introduced by site-directed mutagenesis. A normal PCR reaction was performed by using 10 ng template plasmid DNA, 100 ng each of forward and reverse primer shown with mutated

triplets underlined (Table 2-6). The PCR was performed with Phusion high-fidelity DNA polymerase (Finnzymes) using HF buffer according to the manufacturer's instructions for 12 cycles. The PCR product was treated with 5U of *DpnI* restriction enzyme to digest wild-type plasmids used as template. The resulting plasmids were transformed in the *E. coli* strain XL1 blue and minipreps were made from 5 ml overnight cultures obtained from selected colonies. The resulting plasmids were sequenced to confirm mutations and valid clones were transformed in the *E. coli* expression strain M15[pRep4] for subsequent protein expression and purification.

Table 2-6 Primers used to generate Cys-Ser mutants of SCE1.

Mutant SCE1		Primers
C44S	F	CTAATGGTGTGGCATAGCACTATACCTGGTAAA
	R	TTTACCAGGTATAGTGCTATGCCACACCATTAG
C76S	F	AGCAAACCTCCGAAAAGTAAATTTCCACAAGGG
	R	CCCTTGTGGAAATTTACTTTTCGGAGGTTTGCT
C94S	F	CCATCTGGAACTGTCAGTCTCTCTATCCTTAAC
	R	GTTAAGGATAGAGAGACTGACAGTTCCAGATGG
C139S	F	GGTTATCATCTCTTCAGTCAGGATCCAGTTGAG
	R	CTCAACTGGATCCTGACTGAAGAGATGATAACC

2.8.4 Overproduction of recombinant proteins in *E. coli*

E. coli expression strain M15[pRep4] was transformed by the heat-shock method. The resulting colonies were selected on LB plates having 25 mg/l kanamycin to select for repressor plasmid pRep4 and 100 mg/l ampicillin to select

for pQE70 expression plasmid. The colonies were grown overnight in 5 ml LB including antibiotics and sub-cultured in 100 ml LB media with antibiotics. The cultures were grown at 37 °C for a few hours until OD_{600} reached 0.7 – 0.8. Protein expression was induced by adding IPTG (Isopropyl β-D-1-thiogalactopyranoside) to a final concentration of 1 mM. To enhance solubility, the induction was carried out at 30 °C for 5 hrs. The induced cultures were pelleted by centrifugation at 4500*g* for 15 min. The harvested cells were stored at −80 °C for subsequent protein purifications.

2.8.5 Protein purification from induced *E. coli* cultures

The following buffers were used to purify native recombinant proteins from induced *E. coli* pellets.

Lysis buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole pH = 8
Wash buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole pH = 8
Elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 400 mM imidazole pH = 8

Hexa-His-tagged proteins were purified by affinity chromatography using Ni-NTA spin kits (Qiagen) with several modifications to the recommended procedures. The pellets from 100 ml induced cultures were resuspended in 1 ml lysis buffer and supplemented with 1 mg lysozyme, 300U of Benzonase Nuclease® and 1 mM PMSF (phenylmethylsulfonyl fluoride) and 0.25% Triton X-100. The suspensions were shaken for 30 min at room temperature and imidazole added to 10 mM after lysis. The mixtures were centrifuged at 4 °C to clear debris and clear lysate was filtrated through 0.45 μm pore-size syringe filters (Millipore) using 5 ml disposable syringes. The lysates were passed through pre-equilibrated Ni-NTA spin columns by centrifugation at 250*g* for 2-5 min. The columns were

washed three-times with wash buffer and 6XHis-tagged proteins were eluted twice with 250 µl elution buffer by centrifugation for 2 min at 500*g*. The protein concentrations were measured by Bradford assay (Bradford 1976) using BSA as standard. The proteins were dialysed overnight against the desired buffers to remove imidazole before these were used in different assays.

2.8.6 Antibody production

Synthetic peptides for SUMO1/2 (CSANQEEDKKPGDGGAH), SUMO3 (CNPQDDKPIDQEQEAH) and SUMO5 (CFVSKKSRSPETSPHM) conjugated to KLH (keyhole limpet hemocyanin) for immunization and antibody production were ordered from JPT peptide technologies, GmbH, Germany. Three milligram of each peptide was used to immunize rabbits. Whole antisera were collected and freeze dried into lyophilized powder. Lyophilized serum (50 mg) was dissolved in 500 ml of water and the volume raised to 1 ml by adding glycerol in order to avoid freeze-thaw cycles. The specificity of each antibody was tested for respective peptides and optimum working dilutions were worked out. An IgG (immunoglobin G) HRP (Horseradish Peroxidase)-linked secondary antibody against rabbit Ig was used at 1:2000 dilution for immunodetection. An HRP-linked anti-biotin antibody (Cell Signalling Tech.) was used to detect biotinylated samples according to the manufacturer's instructions.

2.8.7 SDS-PAGE and Western blot analyses

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and western blots were carried out as described by Sambrook & Russel (2001) with slight modifications. Crude protein extracts were supplemented with 4X SDS

loading buffer (250 mM Tris-Cl pH 6.8, 40% glycerol, 8% SDS, 0.04% bromophenol blue) with or without 25 mM DTT. The samples were heated at 70 °C for 15 min and separated by SDS-PAGE at 120 V for ~ 2 hrs. The gels were either stained with Commassie brilliant blue solution (0.25% brilliant blue-R, 40% methanol, 7% acetic acid, 53% water) or the proteins were transferred onto Hybond^{TM}-P PVDF membrane (Amersham Biosciences) in the transblotting buffer (25 mM Tris, 200 mM glycine and 20% Me-OH) at 80V for 1 hr at 4 °C. The membranes were blocked in 25 ml blocking buffer (1X TBS containing 50 mM Tris-Cl pH 7.4 and 150 mM NaCl, 0.1% Tween-20 and 5% non-fat dried milk) for 1 hr on the tilting table at room temperature. The blots were incubated overnight at 4 °C with the respective antibody at optimum dilution in the blocking buffer. Next morning, the membranes were washed 3 times with TBS/T (1X TBS + 0.1% Tween-20) and incubated with IgG HRP-linked secondary antibody for 1 hr at room temperature. The immunodetection was carried out by using Amersham ECL plus western blotting detection kit (GE Healthcare) which uses a non-radioactive chemiluminscent detection reagent. The blots were exposed to X-ray films (CL-XPosure Film, Thermo Scientific, USA) and autoradiographs were developed in an X-ray developer. The Commassie stained gels were washed in destaining solution (10% methanol, 10% acetic acid, and 80% water) and were used as loading controls showing total assayed protein or protein bands were excised for MS analysis.

2.8.8 *S*-nitrosylation assay

Biotin switch technique was used to detect Cys residues modified by the NO donor GSNO or CysNO as described by Jaffrey and Snyder (2001). The procedure replaces NO attached to Cys-thiols with the biotin, which can then be detected by immunoblotting using an anti-biotin antibody. The protein samples (100 µl in volume) having 0.8 µg/µl of purified protein were incubated with 250 µM either GSNO (*S*-nitrosoglutathione), CysNO (*S*-nitrosocysteine) or Glutathion (GSH) which was used as an inactive control. The samples were incubated in the dark for 20 min at room temperature and all the succeeding procedures were carried out away from direct light until biotinylation. The excessive NO donor was removed by passing the samples through Micro Bio-Spin P6 columns (Bio-Rad) pre-equilibrated with HEN buffer (250 mM Hepes-NaOH pH 7.7, 1 mM EDTA, and 0.1 mM Neocuproine). The free –SH groups were blocked with MMTS (S-methylmethanethiosulfonic) blocking buffer (HEN buffer, 2.5% SDS and 20 mM MMTS) at 50 °C for 20 min. The MMTS blocking buffer was removed by acetone precipitation and the protein samples were recovered by dissolving the pellets in 50 µl HENS buffer (HEN buffer + 1% SDS). The samples were incubated with 1 mM each of Biotin-HPDP (N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide) and ascorbate. The reaction was allowed to proceed for 1 hr at room temperature. The samples were supplemented with 4X SDS non-reducing loading buffer, heated at 70 °C for 10 min and separated on SDS gels or stored at 4 °C until the next day. Western blots were performed as described and detection was made by using anti-biotin antibody (1:2000 dilution).

2.8.9 Mass spectrometry (MS) analysis

After the biotin switch assay, *S*-nitrosylated proteins were separated on 12% SDS gels and stained with Coomassie brilliant blue (R). Protein bands were excised from the gels and subjected to tryptic and/or proteinase K digests. LC-MS/MS (liquid chromatography/mass spectrometry/mass spectrometry) was carried out on Thermo LTQ orbitrap (Thermo Electron Corp. CA, USA) according to manufacturer's instructions. The MS/MS spectra were obtained which were further analysed to identify biotinylated cysteine residues in the proteins.

2.9 *In vitro* SUMOylation assay

A standard *in vitro* reaction was setup in buffer containing 50 mM Tris-Cl pH 7.8, 100 mM NaCl, 10 mM $MgCl_2$ and 5% glycerol. Recombinant proteins were dialysed against the reaction buffer before they were mixed together making a total reaction volume of 50 µl. The *In vitro* assay was carried out by mixing 8 µg SUMO1, 1 µg each of SAE1a and SAE1b, 2 µg SAE2, 2 µg SCE1 and 4 µg *Sc*PCNA. The reaction was started by adding 5 mM ATP and incubated at 25 °C for 4 hrs before it was stopped by adding protein loading buffer and heating at 70 °C for 10 min. Samples were separated on 12% SDS gels and SUMO-conjugation was detected by western hybridization described above using anti-SUMO1 primary antibody (diluted 1:2000) and HRP-linked anti-rabbit secondary antibody (diluted 1:2000).

Chapter 3

3 Testing SUMO Machinery Mutants against Pathogens

3.1 Background

Genetic dissection of pathogenicity, host resistance and underlying defence signalling pathways has been immensely facilitated by the unique characteristics of the model plant *Arabidopsis thaliana*, publication of its genomic sequence in 2000 and prompt availability of knockout mutants for almost all the genes. Though our understanding of genes regulating plant disease resistance has been vastly increased during the last decade, the complex post-translational events occurring during multiple signalling pathways and genetic/molecular characterization of these events is still lagging behind. T-DNA insertion mutagenesis in *Arabidopsis* has provided us with a direct route of elucidating the function of such interacting genes by allowing us to abolish their activity in so called knockout lines; the approach commonly known as 'reverse genetics'. An integration of these genetic tools with modern molecular biology and bioinformatic approaches enabled us to study the roles of SUMOylation in plant pathogenesis.

The evidence of the involvement of SUMO in various human diseases like cancer (Kim et al., 2006; Moschos and Mo, 2006), Alzheimer's and type I diabetes (Li et al., 2005), Huntingtin and Huntington's disease (Steffan et al., 2004) the circadian clock (Cardone et al., 2005), and neurodegenerative diseases (Beg and Scheiffele, 2006; Dorval and Fraser, 2007) highlights its significance in

the disease process. Hence, a link between SUMOylation and plant pathogenesis is very likely. Further, it may also increase our understanding of SUMOylation in animal biology as there are certain restrictions and limitations to experiment with animals from which plants are exempt.

3.2 Functional Genomics of SUMOylation

3.2.1 Microarray gene expression data mining

Gene chip technology now enables us to profile the expression of several thousand genes and these datasets are now available publically. From such online resources (www.arabidopsis.org), the microarray data mining for SUMO genes, their activation, conjugation and ligation counterparts and SUMO proteases in *Arabidopsis* suggests that these genes are expressed in almost all plant parts throughout the life cycle. The expression patterns, however, vary substantially from organ to organ and most of the proteins are relatively highly expressed in growing buds and apical meristems and moderately expressed in leaves.

The expression pattern analysis from *Arabidopsis* eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) which creates an 'electronic fluorescent pictographic' representation of the gene of interest's expression patterns revealed the highest expression of *SUMO1* and *SUMO2* with mRNA level of around 10-times greater than *SUMO3* and *SUMO5* (Table 3-1). *SUMO4* expression was found 100 times less than *SUMO1* and *SUMO2* (Table 3-1). There is no microarray gene expression data available for *SUMO6, SUMO7* and *SUMO8*. Among all the SUMO proteins, SUMO1 and SUMO2 are highly similar

(94% amino acid sequence identity) (Fig 3-1), are functionally redundant and their double mutants are lethal (Saracco et al., 2007; van den Burg et al., 2010).

Likewise, the gene expression profile for E1 enzymes (*SAE1a, SAE1b, SAE2*) revealed the mRNA levels as the same for both *SAE1a* and *SAE1b* but 3-4 times higher than *SAE2*. High amino acid similarity between SAE1a and SAE1b (81%) and their similar gene expression levels suggest these genes might be functionally redundant as knocking out one does not contribute to any phenotypic effect, which would have, otherwise, been lethal as SUMOylation is essential for normal cellular affairs.

Table 3-1 Relative expression profile of SUMO machinery genes.

Gene	Relative expression in leaves	Fold change in expression after pathogen challenge		
		B. cinerea **(48 hpi)**	***Pst*DC3000 (24 hpi)**	***Pst*DC3000(*avrRpm1*) (24 hpi)**
SUMO1	500	0.69	0.68	0.81
SUMO2	400	0.78	0.74	0.64
SUMO3	58	0.56	0.74	no change
SUMO4	4	1.35	2.73	2.87
SUMO5	50	0.63	0.22	0.43
SAE1a	160	0.75	0.57	0.73
SAE1b	150	no change	1.55	0.78
SAE2	40	1.27	1.94	1.77
SCE1	1000	0.86	0.87	0.92
SIZ1	33	no change	0.89	0.83
UPL1b	2	0.69	1.12	0.91
ULP1c	50	0.6	0.54	0.76
ULP1d	140	1.28	0.82	0.88
ESD4	70	1.15	1.16	0.19

Presence of only a single SAE1 subunit in mammals (known as Aos1) is sufficient to carry out SUMOylation. The accumulation of *SAE1a* and *SAE1b* in equal stoichiometric ratios is also obvious from their gene expression profile (Table 3-1). *SCE1* is the highest expressing gene amongst all the SUMO components with relative expression level 2-3 times higher than the most highly expressed SUMOs (*SUMO1* and *SUMO2*), and 7-10 times higher than E1 enzymes. *SCE1* is consistently expressed in all tissues throughout the life cycle but in germinating seeds where the expression level is marginal.

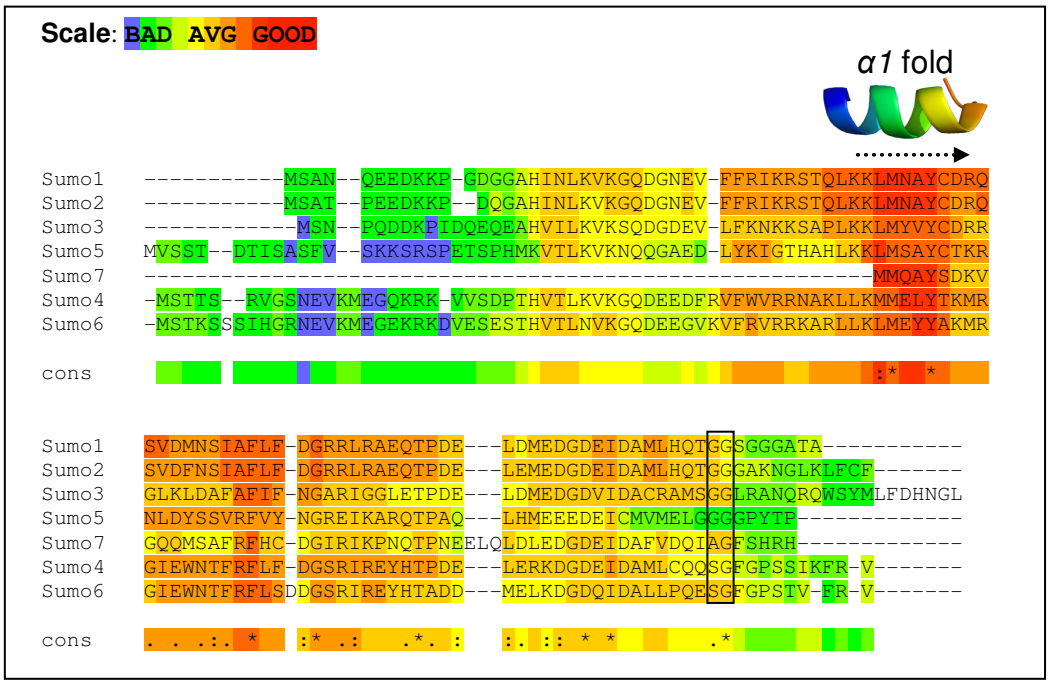

Figure 3-1 Amino acid sequence alignment of SUMO1-7 proteins

High homology region is shown with dotted lines which constitutes an *α1* helix segment (generated by PyMoL™, DeLano Scientific LLC) within a $\beta_1\beta_2\alpha_1\beta_3\beta_4\alpha_2\beta_5$ fold. A mono or di-glycine cleavage of precursor SUMO into a mature form is represented by a line box. The sequence analysis was carried out online using TCoffee webserver (www.igs.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi).

The microarray data mining for SUMO proteases revealed the highest relative expression of *ULP1c* and *ULP1d* followed by *ESD4* and *SIZ1*, while *ULP1b* transcript levels were about 50-times less than the average expression of *ULP1c* and *ULP1d*. *SIZ1* is the best characterized gene amongst the SUMOylation components and is regarded as an important player in stress signalling, particularly, plant defence. Interestingly, the expression of *SIZ1* is the highest in senescent leaves and dry seeds, while *SIZ1* is equally expressed in all plant parts untill flowering. Unexpectedly, the biotic stress data suggests only a slight decrease in *SIZ1* expression after *Pst*DC3000 infection (Table 3-1).

3.2.2 Changes in gene expression after pathogen challenge

The eFP browser biotic stress profile (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi? dataSource=Biotic_Stress) portrays a slight reduction (1-2 times) in the expression of *SUMO1, 2* and *3* after *B. cinerea*, *Pst*DC3000 and *Pst*DC3000 (*avrRpm1*) challenge except for *SUMO4*, where the mRNA transcript level is up-regulated 2-3 times. Interestingly, the expression level reduces 3-5 times in case of *SUMO5* suggesting SUMO5 could be important in defence regulation. Further data mining from the online resources regarding E1 and E2 enzymes revealed only slight changes in mRNA transcript levels after pathogen infection. Furthermore, the expression of *ULP1c* is significantly down-regulated after *B. cinerea* and *Pst*DC3000 challenge.

3.3 Genotyping to Identify Homozygous Lines

A line without a T-DNA insertion at either of the chromosome in the homologous pair gave a wild-type band (~ 1 kb) after gel electrophoresis in both

sets of PCR, while the line with a T-DNA insertion on both homologous chromosomes and no wild-type gene copy only amplified a ~ 0.5 kb fragment when T-DNA-specific primer was used in combination with gene-specific primers. While the genotypes having a single T-DNA insertion on one of the homologous chromosomes gave two distinct bands (wild-type and T-DNA insert) after the PCR products were separated on 1.5% agarose gels (Fig 3-2). In this way, about 35 homozygous mutant lines were isolated for 14 SUMO machinery genes listed in Table 2-1 in the previous section and the seeds were collected.

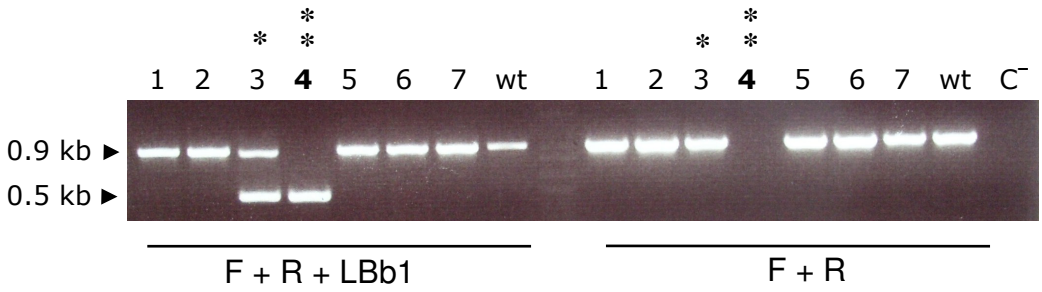

Figure 3-2 A gel picture showing T-DNA mutant genotyping of a SALK line.
Two PCRs were performed using gene-specific primer pair (right panel) and an additional T-DNA specific left-border primer (left panel). Lane # 3 giving two bands (a wild-type and a T-DNA insert) is designated as a heterozygous line shown by single asterisk (*), while line # 4 yielding only a single ~0.5 kb band was a homozygous line for T-DNA insertion and is shown by double asterisks (**). C^- is a negative PCR control and wt designates Col-0 gDNA. Arrow heads show approximate DNA fragment sizes.

3.3.1 Gene knockout confirmation

In order to confirm if T-DNA insertion was sufficient to abolish transcription, total RNA was isolated from the leaf tissues of mutants identified as homozygous and subjected to reverse transcriptase (RT)-PCR or northern blot analyses. In case of *SUMO1*, *2*, *3* and *5*, high gene sequence homology and

smaller gene sizes was a major constraint to generating gene-specific probes which could be used for northern blot analysis. Hence, RT-PCR analysis was the method of choice where unique 5′ or 3′ untranslated regions (UTRs) were used to design primers and amplify first-strand cDNA generated in RT reactions. These analyses led to the identification of lines where gene expression was abolished by T-DNA insertion. These lines were designated as knockout or null-mutants and were the subject of further disease assays.

The RNA analyses indicated that T-DNA insertion does not always abolish transcription. The gene expression level may be reduced rendering a knockdown mutant, or may remain unchanged or in rare cases may be upregulated if the insert is in the promoter region. As our primary goal was to track down complete loss-of-function mutants, a complete absence of transcript was the desired situation. Absence of a transcript signal in RT-PCR analysis carried out for *sumo1-2* (GABI_675B02) confirmed its loss-of-function and the line was designated as a null-mutant (Fig 3-3 A). Null-mutant for *SUMO2* (SALK_129775) and a knockdown mutant for *SUMO3* (SALK_123673) have already been identified (Saracco et al., 2007). Surprisingly, after analysing five T-DNA mutants for *SUMO5* (*sumo5-1*; GABI_606E01, *sumo5*-2; SALK_119313C, *sumo5-3;* SAIL_770_G01, *sumo5-4*; SALK_085812 and *sumo5-5*; GABI_370D01) the presence of transcript signal after RT-PCR revealed none of them was a knockout allele. Interestingly, an overexpression signal for *sumo5-4* (SALK_085812) was obvious (Fig 3-3 B). An extremely low expression of SUMO4, 6 and 7 was a major constraint in amplifying the full-length cDNA from

the total RNA preps using standard RT-PCR procedures. Lack of expression data for these genes also supports this argument. It seems like these genes express only under specific circumstance or in specific tissue at certain developmental stages similar to human *SUMO4* which is expressed in kidney cells and lymph nodes and the expression level is many-fold less than the rest of the SUMO genes.

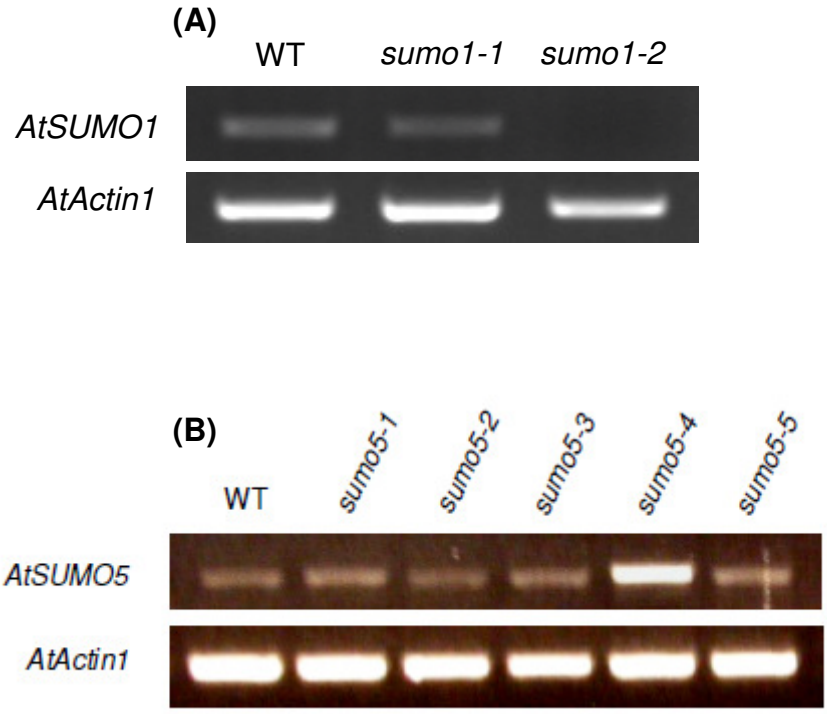

Figure 3-3 Reverse transcriptase (RT)-PCR analysis for gene knockout confirmation.

RT-PCR analysis was performed using total RNA from putative null-mutants and the PCR products were separated on 1.5% agarose gels containing ethidium bromide and photographs taken under UV transilluminator. *Arabidopsis* (*At*) *Actin1* primers were used to validate equal quantities of cDNA. **(A)** The absence of any transcript signal in lane 3 (*sumo1-2*) is suggestive of the line being a null-mutant while *sumo1-1* gives a signal like Col-0 (WT). **(B)** No null-alleles were recovered for *sumo5* T-DNA lines while a relatively bright band in lane 5 (*sumo5-4*) where the T-DNA insert is in the promoter region suggests a higher gene expression compared to the WT.

Northern blot analyses were performed where it was feasible to generate gene specific probes. For *SAE2*, a knock-down mutant was isolated and named *sae2-3* (SALK_094819) (Fig 3-4, A). Importantly, a loss-of-function mutagenesis in *SAE2* has been reported as lethal (Saracco et al., 2007), nonetheless, this mutant lines shows stunted growth, small leaves and a bushy appearance (data not shown). It was viable probably due to a sufficient transcript level required for its survival.

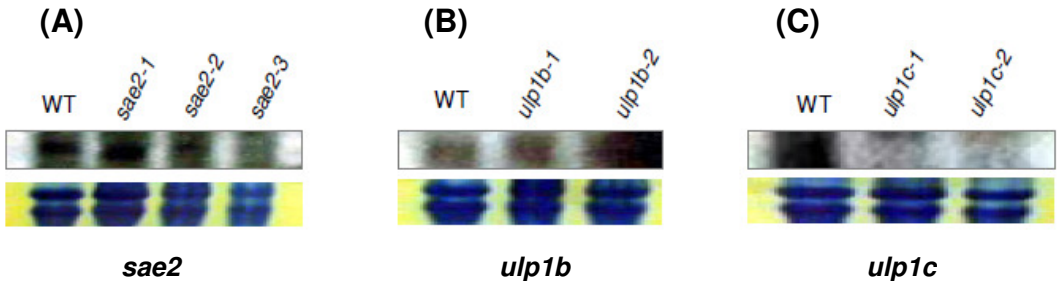

Figure 3-4 Northern blot analysis for gene knockout confirmation.
Total RNA (10µg/lane) was extracted from leaf tissues and hybridized with the probes indicated. Lane1 in each blot corresponds to wild-type Col-0 total RNA. Loading controls (methylene blue stained blots) are shown at the bottom of each figure. **(A)** Three mutant lines for *SAE2* were tested. A faint signal in lane 4 (*sae2-3;* SALK_094819) is suggestive of a knocked down expression. However, no mutant was isolated as a complete knockout. **(B)** No null-mutant was recovered as the transcript level remains almost the same in both lines compared to wild-type Col-0. **(C)** The absence of transcript signal for SALK_151423 (*ulp1c-1*) in lane 2 and SALK_050441 (*ulp1c-2*) in lane 3 shows an abolished gene expression, hence, these lines were designated as null-mutants for ULP1c and named *ulp1c-1* and *ulp1c-2*.

The *ulp1b* mutant line tested through northern blot analysis showed unaltered gene expression designating these mutants as not useful (Fig 3-4, B). However, absence of a transcript signal in two independent lines was noted for

ulp1c (*ulp1c-1*; SALK_151423 and *ulp1c-2*; SALK_050441) rendering these mutants as null-alleles (Fig 3-4, C). The mutant lines were tested in pathogenicity assays in compatible and incompatible pathogen interactions with *Pst*.

3.4 Disease and HR response

3.4.1 SUMO mutants showed moderate resistance against *Pst*DC3000

A reverse genetic approach was employed in order to determine if any component(s) of SUMO machinery is/are involved in disease resistance. Four-week old plants were syringe infiltrated on the abaxial side of the leaves with virulent bacterial pathogen *Pst*DC3000 suspension. Disease symptoms were recorded 3-4 days post inoculation (dpi) and bacterial growth was measured by colony counts. Col-0 was used as a wild-type control while *S*-nitrosoglutathione (GSNO) reductase loss-of-function mutant (*atgsnor1-3*) was used as a susceptible control (Feechan et al., 2005). A moderate reduction in bacterial growth was observed in *sumo1-2*, *sumo2-1*, *sae1a-1* and *sae2-3* compared to wild-type after DC3000 infection, however, the differences were not statistically significant (Fig 3-5). This was further confirmed by visually scoring disease symptoms where only minor differences were seen (Fig 3-6). Taking *sumo1* and *sumo2* into account first, the lack of any phenotype might be due to their functional redundancy, since the double mutants have been reported as lethal (Saracco et al., 2007). The same could be true for *SAE1a* due to its high similarity with *SAE1b* which could not be tested due to its mutant unavailability. It seems likely in the

absence of one paralogue, the other compensates for its function yielding a wild-type phenotype as single gene mutation had no phenotypic effect.

The *sae2-3* mutant was not a complete knockout, however, the plants were stunted and bushy with small leaves (data not shown) and this knockdown mutant showed slightly enhanced resistance against DC3000, although, the bacterial growth was not significantly different from the wild-type plants as revealed by the Student's *t* test.

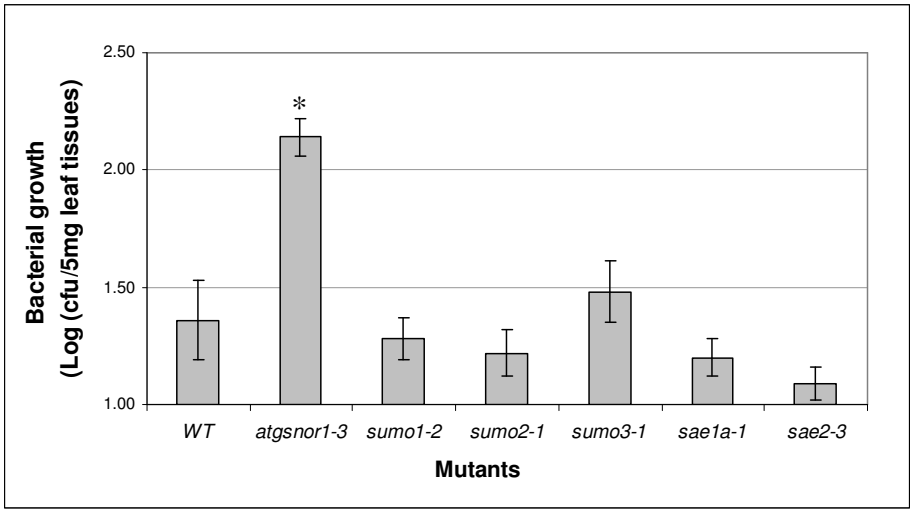

Figure 3-5 Growth of *Pst*DC3000 in various mutant lines.

Plants were infiltrated with a *Pst*DC3000 suspension of 10^5 cfu/ml and colony counts were made 4 dpi. A modest reduction in *Pst*DC3000 growth was observed in *sumo1-2*, *sumo2-1*, *sae1a-1 and sae2-3* mutant but remained statistically insignificant. Values shown are the mean log of bacterial counts (n = 4) ± S.D. The experiment was repeated twice with similar results. Student's *t* test showed significant difference in mean log bacterial growth at p = 0.05 (*) only in *atgsnor1-3* plants.

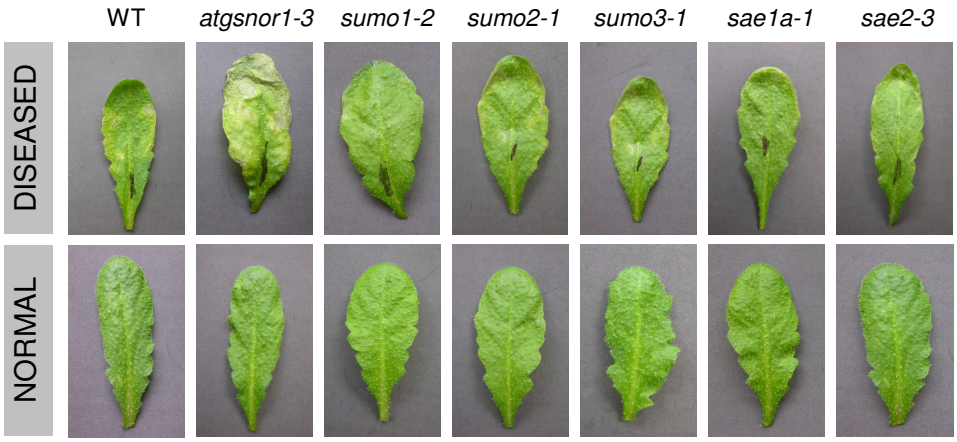

Figure 3-6 Disease symptoms after *Pst*DC3000 infection in SUMO mutants. Plant leaves were infiltrated with a *Pst*DC3000 suspension of 10^5 cfu/ml and disease symptoms recorded 4 dpi. *sumo1-2, sumo2-1*, *sumo3-1*, *sae1a-1* and *sae2-3* show moderate resistance as compared to the wild-type (Col-0). Basal resistance was compromised in *atgsnor1-3* which was used as a susceptible control.

3.4.2 SUMO mutants show modestly reduced HR

In order to determine if SUMO mutants are defective in *R* gene mediated defence, plants were challenged with *Pst*DC3000 expressing *avrB* which is recognized by *Arabidopsis R* gene '*Rpm1*'. The leaves were syringe infiltrated with bacterial suspension set at OD_{600} = 0.02 (10^7 cfu/ml) in 10 mM $MgSO_4$. The electrolytic leakage assay was performed which measures the conductivity of the water containing infiltrated leaf discs. The conductivity increases over time as a result of the ion leakage from the tissues undergoing HR leading to cell death. It was found that *sumo1-2, sumo2-1*, show moderately reduced AvrB mediated HR compared to the wild-type plants, particularly 24 and 48 hpi suggesting a minor role of *SUMO1* and *SUMO2* in *R* gene mediated defence signalling. The mutant *atgsnor1-3* used as negative control shows the highest ion leakage while other

mutant lines did not show substantial differences in ion leakage over-time. To further investigate about SUMO mutants in the defence response, the leaves undergoing HR were subjected to trypan blue staining assay which reveals cell death (Fig. 3-8). The differences observed in ion leakage assays were not adequately confirmed by the TB staining experiments (Fig 3-8).

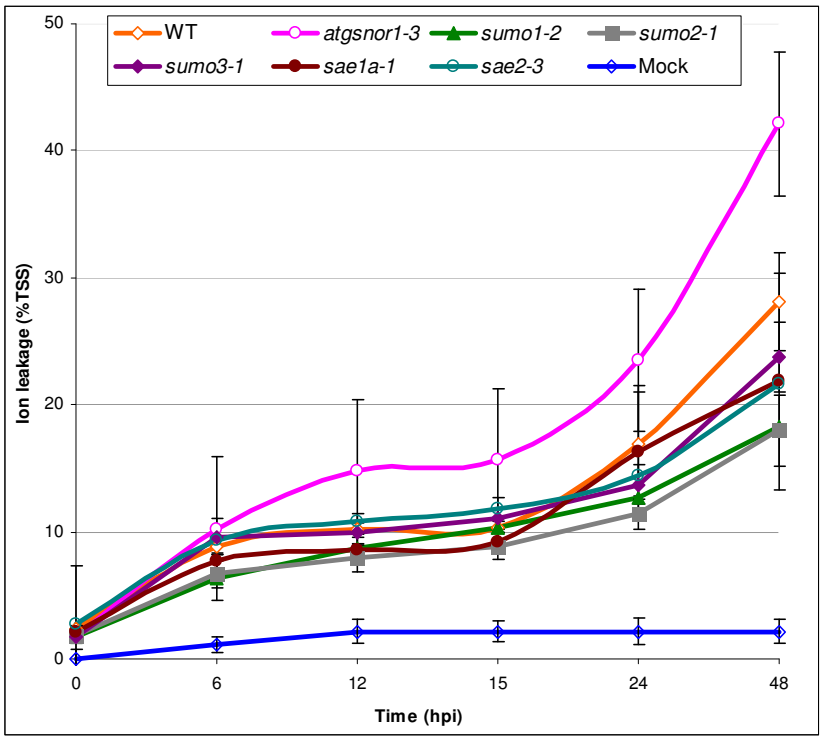

Figure 3-7 Ion leakage assay to quantify HR after *Pst*DC3000(*avrB*) challenge. Leaves were infiltrated with a *Pst*DC3000(*avrB*) suspension set at OD_{600} = 0.02 (10^7 cfu/ml). Leaf-discs of 1 cm^2 were placed in small trays having 10 ml water. Conductivity was measured at the given intervals and expressed as percentage of total ions present in the leaf-discs. *sumo1-2, sumo2-1*, *sumo3-1*, *sae1a-1* and *sae2-3* show moderately low ion leakage compared to the wild-type, particularly, 24 and 48 hpi. *atgsnor1-3* used as negative control showed the highest ion leakage as expected. Each experiment was replicated three-times and error bars show standard error (SE).

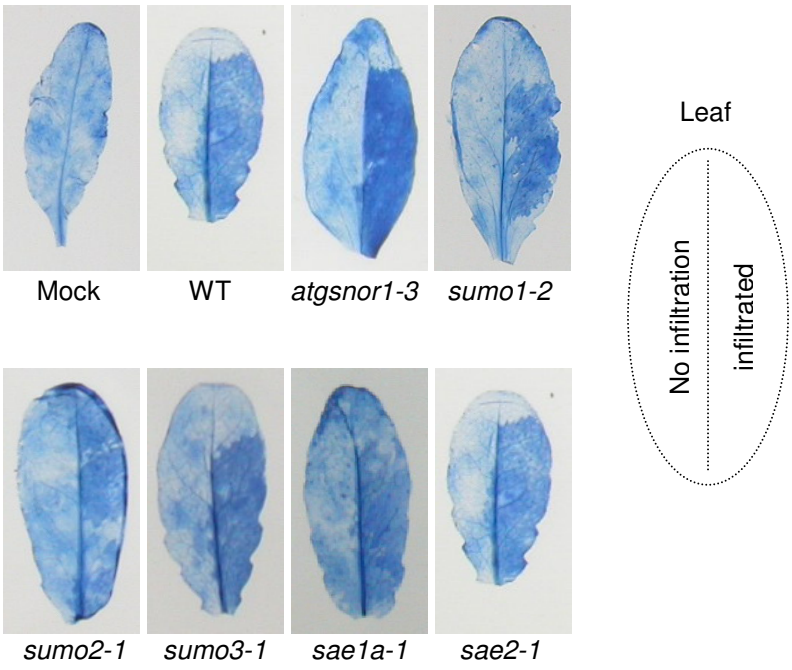

Figure 3-8 Trypan blue staining to measure cell death.

Leaves were syringe infiltrated with a *Pst*DC3000(*avrB*) suspension set at OD_{600} = 0.02 (10^7 cfu/ml) and samples were taken at 18 hpi. Staining was performed by dipping the leaves of genotypes listed in trypan blue solution for 5 min followed by de-staining using chloral hydrate solution. No noticeable difference was seen in all the mutants compared to wild-type. *atgsnor1-3* used as negative control shows dark staining suggesting more cell death occurred at 18 hpi. Each leaf is representative of its respective mutant line.

3.5 Discussion

The functional genomics of *Arabidopsis* suggests the occurrence of widespread SUMOylation throughout the plants' life-cycle and apparent changes in *SUMO* genes expression during a variety of different stress cues. The presence of eight *SUMO* genes in *Arabidopsis* highlights its more pronounced role in plants as compared to mammals where the number is only four. In contrast to mammals, links between SUMOylation and plant disease resistance are not well explored.

Apart from some preliminary reports on SUMO proteases and ligases, SUMO genes are least studied in plants. No data have been available since the first published report on the involvement of T-SUMO interacting with ethylene inducing xylanase (EIX) from the fungus *Trichoderma viridae* which causes rapid induction of plant defence response in tomato (Hanania et al., 1999). After more than a decade, van den Burg and co-workers (van den Burg et al., 2010) studied the role of SUMO1, 2 and 3 in plant development and defence against pathogens and proposed that both high and low levels of SUMOylation render defence activation. They suggested that tight transcriptional regulation of SUMOylation is fundamental to the normal cellular affairs and defence against pathogens. Moreover, functional redundancy of SUMO genes remained a major concern in using reverse genetic approach which remained a constraint in the present study as well.

To find and elaborate any role(s) of SUMOylation in plant disease development process and plant defence response, reverse genetic screening of T-DNA insertion mutants for the SUMOylation machinery was carried out. The knockout and knockdown lines identified were subjected to disease and HR assays in order to examine if any of the SUMO components are involved in plant defence response. A slight decrease in susceptibility was recorded for *sumo1-2*, *sumo2-1*, *sae1a-1 and sae2-3* however, compared to the wild-type, the differences were not statistically significant to designate any of these mutant lines as more susceptible. A similar picture was portrayed in incompatible pathogen interactions where *Pst*DC3000(*avrB)* challenged plants displayed slightly reduced cell death

compared to the wild-type. Apparently, no striking differences could be detected after trypan blue staining of leaves undergoing HR. This might be due to gene functional redundancy which is an important feature of higher organisms and is a major constraint in using reverse genetic approaches. SUMO1 and 2 proteins being the most important SUMOs are 97% similar when mature and are functionally redundant. Their double-knockout mutants are lethal (Saracco et al., 2007), while single gene mutation does not have a visible phenotypic effect. The same could be true regarding SAE1a and SAE1b where high protein sequence identity (81%) may results in functional redundancy and lack of any phenotype.

Chapter 4

4 *P. syringae* Subvert Protein SUMOylation in Plants

4.1 Background

The post-translational modifier SUMO regulates a wide array of biological process in eukaryotes by covalent attachment with other proteins modulating their conformation, function, localization and stability. Widespread SUMOylation has been reported in plants undergoing abiotic stresses like high temperature, H_2O_2, canavanine and ethanol suggesting SUMOylation plays important regulatory roles in stress signalling. Despite a wealth of information on the involvement of SUMOs in various human diseases, the role of SUMOylation in plant pathogenesis is not well explored. The fact that animal and plant pathogens follow very similar strategies to colonize and infect their hosts, and a human pathogen *Lysteria monocytogenes* have been shown to impair protein SUMOylation upon infection (Ribet et al., 2010). It can be speculated that pathogen invasion might regulate protein SUMOylation also in plants.

A change in the cellular redox status is a key event after pathogen invasion where protein *S*-nitrosylation plays a fundamental role. Previously, Feechan and co-workers (Feechan et al., 2005) have shown that mutant *Arabidopsis* plants deficient in an enzyme responsible for cellular GSNO turnover named *Arabidopsis* GSNO reductase (*At*GSNOR) have high cellular *S*-nitrosothiol (SNO) levels both in the presence and absence of pathogens. This results in elevated protein *S*-nitrosylation which leads to compromised defences against pathogens.

Enzymes of the SUMO machinery possess key Cys residues at their active sites and may therefore be targets of *S*-nitrosylation which may regulate protein SUMOylation in plants. Our primary objective was to test if elevated cellular SNO levels have any impact on global protein SUMOylation *in vivo* after pathogen invasion and how these changes are comparable to the infected wild-type plants.

Data from mammalian research suggest that certain pathogen proteins need to be SUMOylated or bacterial virulence factors can deSUMOylate host proteins in order to aid pathogenicity. For instance, tomato-SUMO (T-SUMO) has been shown to interact with a fungal (*Trichoderma viridae*) ethylene inducing xylanase (EIX) leading to low ethylene production and reduced cell death during fungal invasion (Hanania et al., 1999). Certain viruses have also been reported to interfere with host SUMOylated proteins for successful infection. For instance, a vaccinia virus protein A40R is SUMO-modified before its nuclear localization where it facilitates the viral genome delivery into the nucleus and, eventually, to the DNA replication sites (Schramm and Locker, 2005). A bacterial type-III effector YopJ (*Yersinia* outer-protein J) which acts as a SUMO specific protease blocks MAPK signalling cascade and activation of a cell death controlling transcriptional factor NF-κB in mammals by disrupting SUMOylation. Similar effects were reported in tobacco by YopJ-like effector AvrBsT from *X. campertstris* which was found to disrupt SUMOylation to cause pathogenicity. Hence, a weakened immune response in mammals and reduced HR in tobacco is caused by these Ub-like protein proteases (Orth et al., 2000; Orth, 2002).

Reduction in SUMO-conjugation and proteosome independent degradation of Ubc9 to prevent further SUMOylation by a human pathogenic bacteria *Listeria monocytogenes* to facilitate infection has recently been reported (Ribet et al., 2010). On the other hand, overexpressing SUMO1 or 2 in HeLa cells significantly reduced bacterial growth which correlates with increased SUMOylation and elevated resistance (Ribet et al., 2010).

Similarly, a bacterial type-III effector XopD (*Xanthomonas* outer protein D) is a plant specific SUMO protease which can restrain host defences by inhibiting SA and JA induced defence activation by protein deSUMOylation (Hotson et al., 2003; Chosed et al., 2007; Kim et al., 2008; Kay and Bonas, 2009). A *Xanthomonas* YopJ-like effectors AvrXv4 has been shown to have SUMO isopeptidase activity during infection (Roden et al., 2004). These data imply that virulent pathogens have devised strategies to manipulate host SUMOylation to aid host colonization. Furthermore, increased resistance of *Arabidopsis* plants deficient in SUMO specific E3-ligase *siz1* and constitutive expression of SAR in the absence of pathogens in *siz1* plants suggest that SUMOylation may down-regulates defence activation in plants. Importantly, *siz1* plants challenged with *Pst*DC3000 expressing *avrRps4* and not *avrRpm1* were found to be more resistant. This implies that *SIZ1* suppresses *EDS1/PAD4* dependent SA accumulation by facilitating SUMOylation of certain unknown targets and the signalling is accomplished through TIR-NBS-LRR type of R proteins (Lee et al., 2007).

Data from the previous reports highlight various indirect roles of SUMOylation in plant defence signalling but a fundamental question of exactly

how pathogen interactions orchestrate host SUMOylation is still open. In this chapter we have demonstrated the changes in global SUMOylation levels in wild-type and *atgsnor1-3* plants after *Pst*DC3000 and *Pst*DC3000(*avrB*) challenge. Further, we have provided evidence that *S*-nitrosylation is an important mechanism regulating protein SUMOylation in normal as well as infected plants. Our data further highlights the impact of virulent and avirulent *Pst*DC3000 pathogens on SUMO1/2, SUMO3 and SUMO5 conjugation to the cellular proteins which was significantly influenced by the presence or absence of GSNOR1 enzyme.

4.2 *Pst*DC3000(*avrB*) Induces SUMOylation

Considering previous observations that stress induces SUMOylation, we determined if the global levels of SUMO-conjugation and free SUMO accumulation are changed during an incompatible pathogen interaction in wild-type plants. We also tested *in vivo* if *S*-nitrosylation plays any regulatory role in SUMOylation during *R* gene mediated defence responses. For this purpose, we used mutant plants deficient in GSNOR which result in high global endogenous SNOs due to elevated *S*-nitrosylation of cellular proteins. The plants were syringe infiltrated with *Pst*DC3000(*avrB*) bacterial suspension set at 10^7 cfu/ml and total protein extracts were made from the infected leaf samples at the time points given. The levels of SUMO-conjugation and free SUMO accumulation were monitored in immunoblot assays using anti-SUMO1, anti-SUMO3 and anti-SUMO5 antibodies. SUMO1 and SUMO2 are extremely similar, thus anti-SUMO1 antibody can not discriminate between these proteins. Hence, SUMO1 and

SUMO2 isoforms were considered as the same entity and written as SUMO1/2 in the text.

4.2.1 SUMO1/2-conjugates and free SUMO levels increase in *atgsnor1-3* plants following AvrB recognition

Western blot analyses revealed that the basal level of SUMO1/2 conjugates is higher in unchallenged *atgsnor1-3* compared to the wild-type plants. Moreover, SUMO1/2 conjugation and free SUMO accumulation increased following pathogen recognition in *atgsnor1-3* during incompatible pathogen interactions with *Pst*DC3000 expressing AvrB (Fig 4-1). In wild-type plants, however, no substantial change in SUMO conjugation as well as free SUMO accumulation was observed after AvrB recognition (Fig 4-1).

These findings highlight an important regulatory role of *S*-nitrosylation regulating SUMOylation *in vivo* after the activation of defence response triggered by a *CC-NBS-LRR* type of *R* gene which leads to HR and cell death. This increase in SUMO-conjugates was more dramatic at 24 and 48 hpi compared to the control plants designating 0 hpi (Fig 4-1). Yet the level of free SUMO, which runs at ~11 kDa on SDS gels, started increasing particularly at 12 hpi. The free SUMO levels considerably increased upto the maximum recorded time of 48 hpi (Fig 4-1).

It was noticed that the accumulation of high molecular weight (HMW) SUMO1/2 conjugated proteins (> 60 kDa) was higher in *atgsnor1-3*, primarily at 0 hpi where the samples were obtained at the time of pathogen inoculation. In wild-type plants, however, no such HMW SUMO-conjugates were detected even after pathogen infection (Fig 4-1 and 4-4). This indicates that the basal level of

SUMO1/2 conjugation is higher in *atgsnor1-3* suggesting an important regulatory role of *S*-nitrosylation in modulating SUMOylation in plants even in the absence of pathogens.

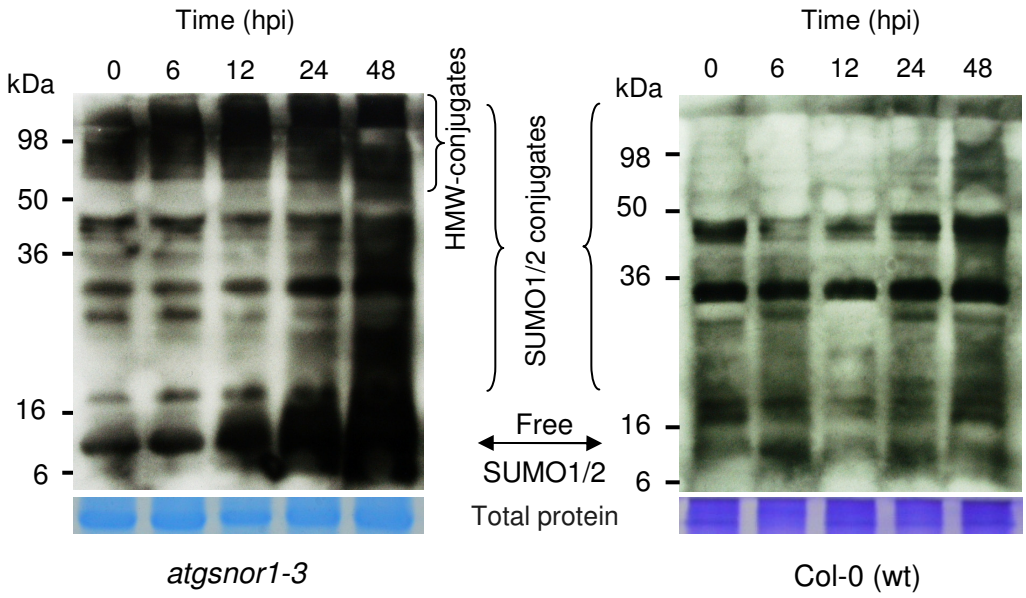

Figure 4-1 SUMO1/2-conjugates increase during *R* gene-mediated defence activation in *atgsnor1-3* plants.

SUMO1/2-conjugation and free SUMO accumulation increased in *atgsnor1-3* **(left panel)**, particularly, at 24 and 48 hpi compared to wild-type plants **(right panel)** during *R* gene mediated defence response leading to HR. The presence of HMW SUMO1/2 conjugates (> 60 kDa) in *atgsnor1*-3 which were not detected in wild-type plants is also evident. Four-week old plants were infiltrated with *Pst*DC3000(*avrB*) suspension set at 10^7 cfu/ml. Total protein extracts were made at time points given. The samples were separated by SDS-PAGE and immunoblot analyses were performed using anti-SUMO1/2 antibody. Coomassie blue stained gels shown at the bottom of each autoradiogram indicate equal protein loading.

4.2.2 SUMO3 dynamics after pathogen recognition and defence response

In order to investigate if SUMO3 levels are modulated after pathogen recognition, western blot analyses were carried out using anti-SUMO3 antibody. It was noticed that an unknown ~10 kDa protein which run at ~20 kDa after SUMO3 attachment was a target of SUMO3 both in wild-type and *atgsnor1-3* plants after *Pst*DC3000(*avrB*) challenge (Fig 4-2). The increase was initiated 6 hrs after infiltration and dramatically increased over time during defence response in both genotypes. Importantly, the free SUMO3 and SUMO3-conjugate levels mostly remained unaltered.

In wild-type plants, a cyclic SUMOylation/deSUMOylation of an unknown ~18 kDa target was also obvious (Fig 4-2) which runs at ~28 kDa on SDS gels due to SUMO3 attachment. However, the dynamics appeared slightly different in both genotypes. Apparently, no remarkable changes were seen for the majority of SUMO3-conjugates suggesting SUMO3 induction is relatively less pronounced during *R* gene mediated defence response compared to SUMO1/2.

The observed increase in the SUMOylation of an unknown ~10 kDa target implies that SUMO3 conjugation to this target protein could be crucial following pathogen recognition. Most of the rest of the SUMOylated proteins virtually yielded the same pattern both in wild-type and *atgsnor1-3* plants on the autoradiogram after western blot analysis (Fig 4-2). Furthermore, the basal levels of SUMO3 and its conjugates were also comparable in both genotypes suggesting *S*-nitrosylation does not regulate SUMO3 modification in contrast to SUMO1/2,

where the effect before and after pathogen treatment remained very striking (Fig 4-1).

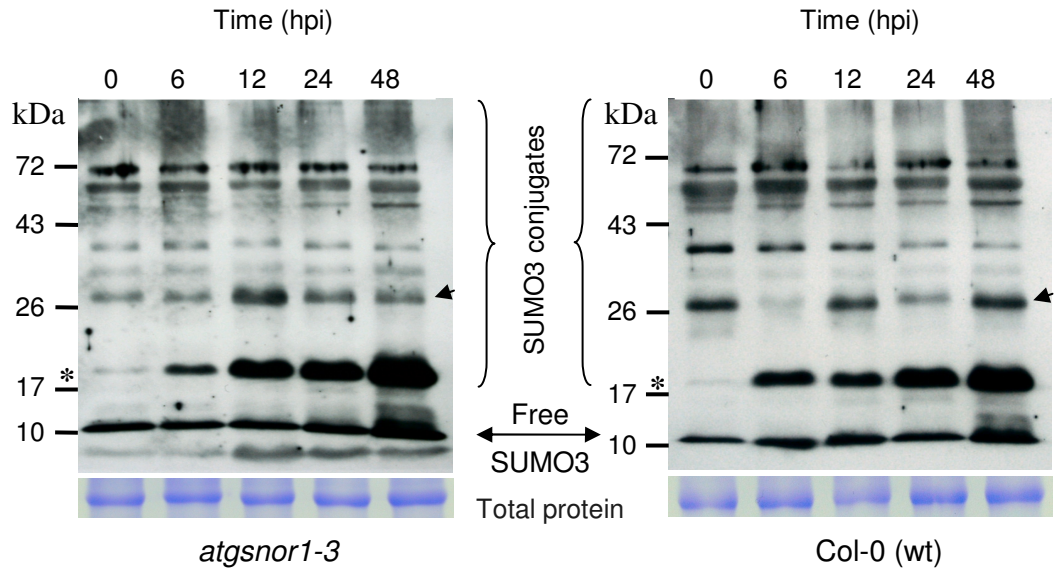

Figure 4-2 Changes in SUMO3 levels following pathogen recognition.
A ~10 kDa protein (shown by an asterisk) was a target of SUMO3 during AvrB-triggered defence both in *atgsnor1-3* and wild-type plants. This unknown target runs at ~20 kDa because of the attachment of a SUMO3 tag. No notable change was observed in overall SUMO-conjugation and free SUMO accumulation except for the ~18 kDa protein (arrow heads) which was SUMOylated/deSUMOylated in a cyclic manner. Four-week old plants were challenged with *Pst*DC3000(*avrB*) suspension set at 10^7 cfu/ml. Total protein extracts were subjected to SDS-PAGE and immunoblot analysis using anti-SUMO3 antibody. Coomassie blue stained gels show equal loading.

4.2.3 SUMO5 levels remain unchanged after *Pst*DC3000(*avrB*) challenge

No noteworthy change in SUMO5-conjugate levels was observed after plants were challenged with *Pst*DC3000(*avrB*). A ~60 kDa protein was targeted by SUMO5 in *atgsnor1-3* plants and not in the wild-type which run at ~70 kDa due to the 10 kDa size shift caused by the SUMO5 attachment (Fig 4-3).

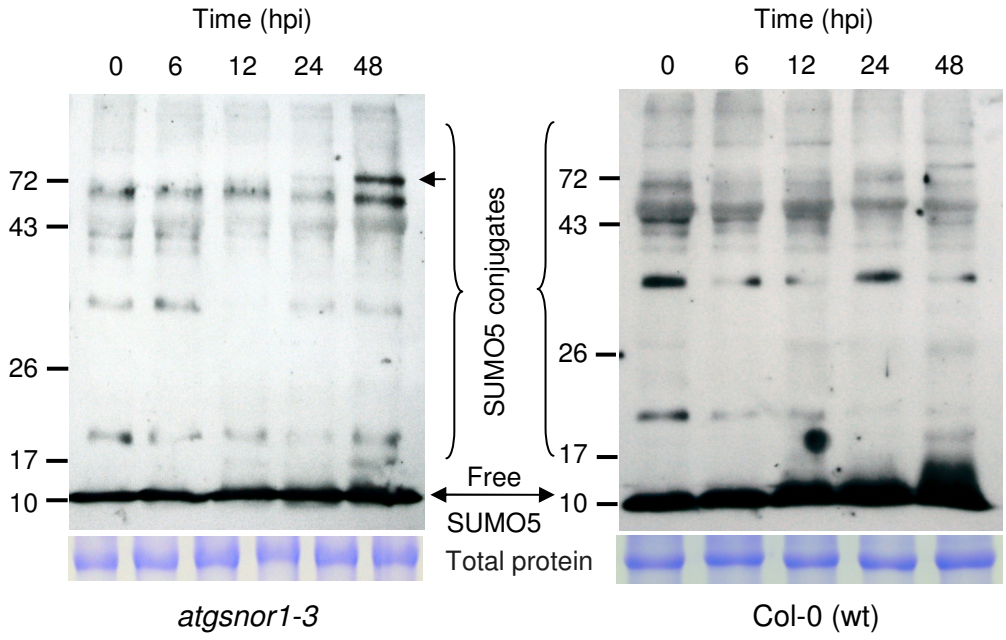

Figure 4-3 SUMO5 levels after *Pst*DC3000(*avrB*) challenge.

A ~60 kDa protein (arrow head) in *atgsnor1-3* which run at ~70 kDa after SUMO5 attachment was found to be SUMOylated after *Pst*DC3000(*avrB*) challenge. No significant changes were seen in overall SUMO5-conjugate and free SUMO5 levels after pathogens infiltration. Four-week old plants were infiltrated with *Pst*DC3000(*avrB*) suspension set at 10^7 cfu/ml. Total protein extracts were separated by SDS-PAGE and subjected to immunoblot analysis using antibody specific to SUMO5. Coomassie stained gels show total protein.

4.3 *Pst*DC3000 Infection Causes Protein deSUMOylation

After demonstrating an increase in SUMO-conjugates in incompatible pathogen interactions where *Pst*DC3000 effector AvrB is recognized by the host R protein Rpm1, the change in SUMOylation level was further trialled in compatible pathogen interactions with *Pst*DC3000 deficient in *avrB* gene, therefore, not recognized in the host cell. This interaction leads to the development of disease due to the absence of *R* gene recognition in the host. Wild-type and *atgsnor1-3* plants were syringe infiltrated with virulent *Pst*DC3000

bacterial suspension set at 10^6 cfu/ml and leaf samples were taken at the given time-points (Fig 4-4). Total protein extracts were separated by SDS-PAGE and western blot analyses were performed using antibodies specific for SUMO1/2, SUMO3 and SUMO5. Levels of SUMO-conjugated proteins and free SUMO accumulation over time after infiltration were compared in wild-type and *atgsnor1-3* plants.

4.3.1 SUMO1/2-conjugates and free SUMO1/2 levels decrease in *atgsnor1-3* during the establishment of disease

A substantial reduction in the global SUMO1/2-conjugates level as well as free SUMO1/2 accumulation was observed in *atgsnor1-3* plants during infection (Fig 4-4). In contrast, no change in SUMOylated proteins was detected in wild-type plants during the establishment of disease (Fig 4-4). These data suggest that virulent *Pst*DC3000 may deSUMOylate cellular proteins to promote infection and this strategy is amplified by high cellular SNO levels. Similar observations have been reported in *Listeria* infection experiments in mammals where a significant reduction in SUMO1/2-conjugates was evident in infected HeLa cells (Ribet et al., 2010). It is important to note that deSUMOylation activity was only manifested in plants deficient in GSNOR and not in the wild-type plants. These additional *in vivo* data lend further support to the notion that *S*-nitrosylation might play an important role in regulating protein SUMOylation after pathogen infection.

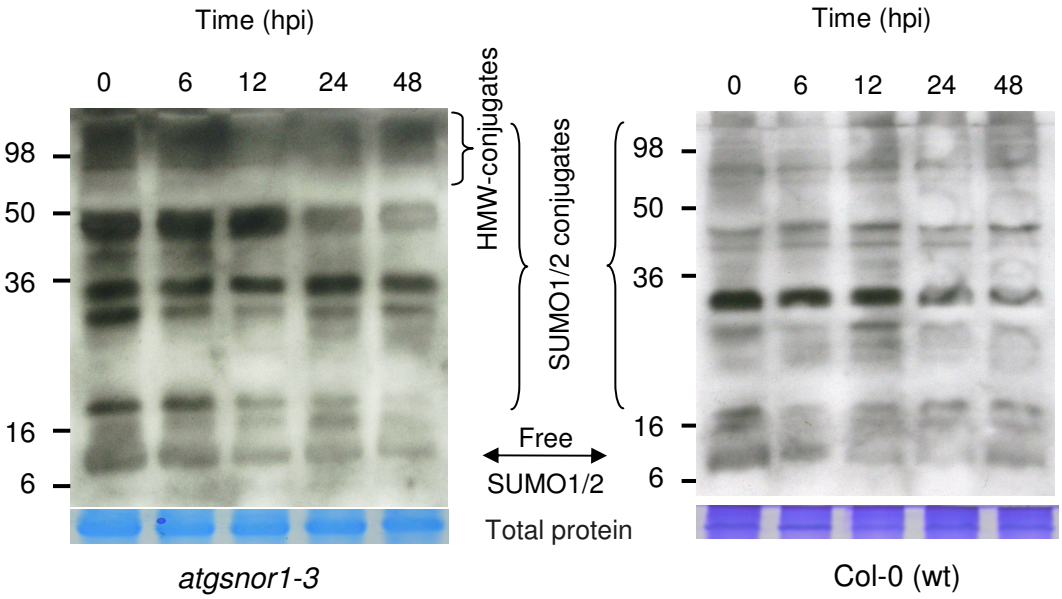

Figure 4-4 Decrease in SUMO1/2-conjugates during *Pst*DC3000 infection in *atgsnor1-3* plants.

atgsnor1-3 mutant plants displayed a decrease in SUMO1/2-conjugation and free SUMO accumulation after virulent *Pst*DC3000 challenge (left panel) compared to wild-type plants (right panel). The basal level of HMW SUMO1/2 conjugates is also higher in *atgsnor1*-3 plants compared to the wild-type. Four-week old plants were challenged with *Pst*DC3000 suspension set at 10^6 cfu/ml. Total protein extracts were made at 0, 6, 12, 24 and 48 hpi and were subjected to SDS-PAGE and immunoblot analyses using anti-SUMO1/2 antibody. Coomassie blue stained gels were used as loading controls shown at the bottom of each autoradiogram.

As observed in avirulent pathogen interactions, the basal level of HMW SUMO1/2 conjugates was much higher in *atgsnor1-3* compared to wild-type plants, particularly at 0 hpi (Fig 4-4). These HMW SUMO1/2 conjugates significantly decreased during the establishment of disease in *atgsnor1*-3. In contrast, the wild-type plants were devoid of such HMW SUMO-conjugates, so as expected, no such decrease was detected even after pathogen infection (Fig 4-4).

These observations imply that basal SUMO1/2 conjugation levels are higher in *atgsnor1-3* suggesting an important regulatory role of *S*-nitrosylation in the regulation of SUMO1/2 conjugation to target proteins even in the absence of pathogen infection.

4.3.2 SUMO3 dynamics after virulent *Pst*DC3000 challenge

An unknown ~10 kDa SUMOylated protein was deSUMOylated during *Pst*DC3000 infection in *atgsnor1-3* plants, which was not very noteworthy in wild-type plants (Fig 4-5). The levels of SUMO3, both free and in conjugated form, remained almost unaltered over time as observed in avirulent pathogen infections.

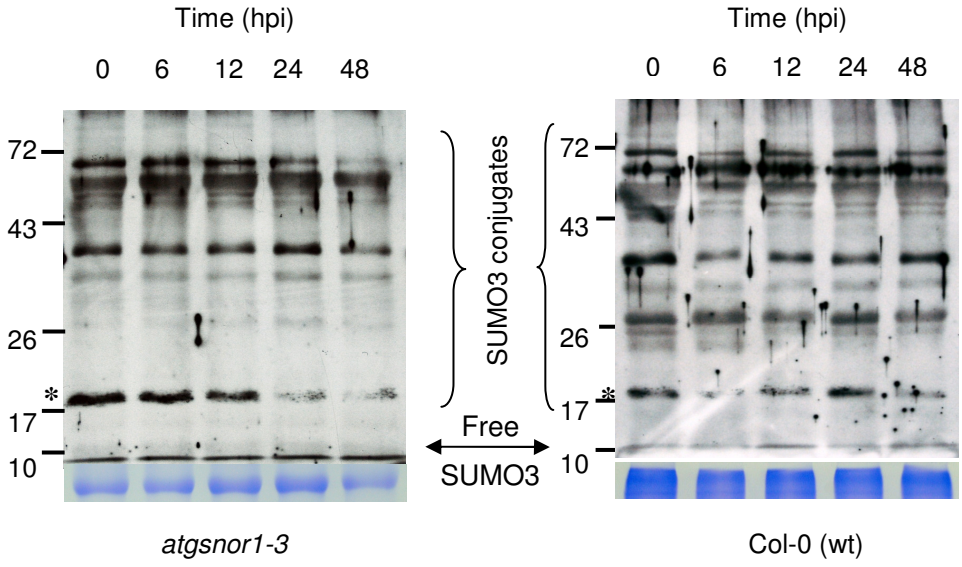

Figure 4-5 Changes in SUMO3-conjugate levels during disease development. The cellular pool of a ~10 kDa protein (shown by an asterisk) is partially deSUMOylated during *Pst*DC3000 infection in four-week old *atgsnor1-3* plants. No noteworthy changes were observed in overall SUMO-conjugate levels. The plants were challenged with *Pst*DC3000 suspension having 10^6 cfu/ml. Total protein extracts were made at time-points given and subjected to SDS-PAGE and immunoblot analysis using anti-SUMO3 antibody. Coomassie blue stained gels represent equal loading.

Keeping in view the previous data (Fig 4-2) where a ~10 kDa protein was extensively SUMOylated following pathogen recognition, the prompt deSUMOylation of the same target during *Pst*DC3000 colonisation suggests that successful pathogens have devised strategies to deSUMOylate certain targets in the host cell in order to cause infection. However, in the presence of an R protein, this unknown target is extensively SUMOylated which could be a result of defence activation. Furthermore, this strategy is facilitated by high cellular SNO levels which, we hypothesize, have a direct effect on SUMOylating enzymes or may impact SUMOylation / deSUMOylation indirectly.

4.3.3 SUMO5 levels largely remain unchanged after virulent *Pst* infections

The levels of SUMO5 conjugates virtually remained unaltered during the establishment of disease after plants were challenged with *Pst*DC3000. Interestingly, a ~60 kDa protein which was SUMO5 modified during the defence response previously (Fig 4-3) was deSUMOylated during the establishment of disease in *atgsnor1-3* plants (Fig 4-6). The blots shown were exposed to the X-ray film for a relatively longer period of time to monitor the changes in SUMO5 modification of this unknown ~60 kDa protein which was made visible even at 0 hpi in lane 1 in *atgsnor1-3* plants (Fig 4-6).

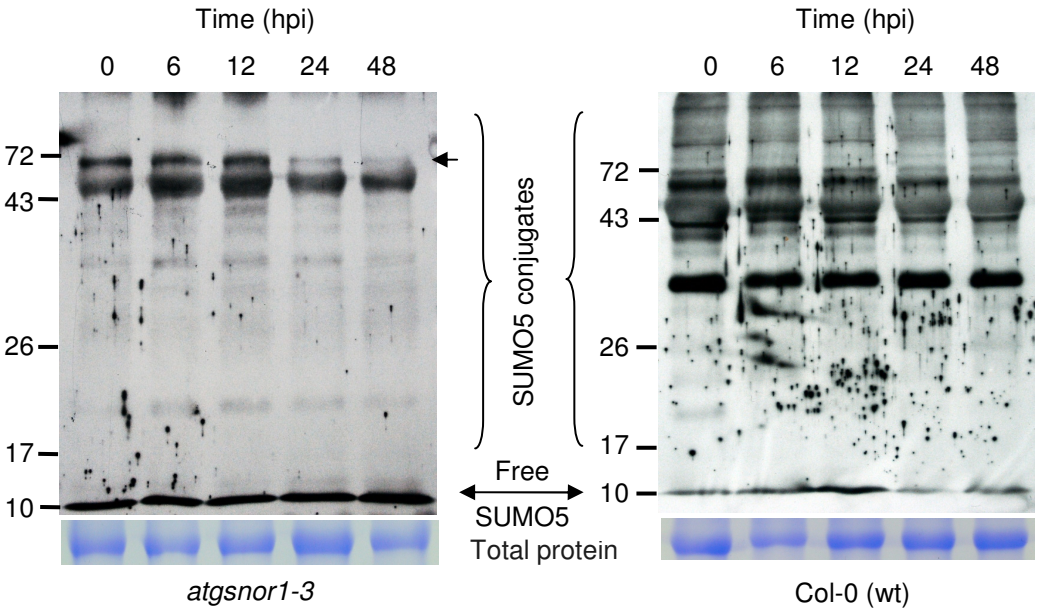

Figure 4-6 SUMO5 levels after *Pst*DC3000 infection.

A ~60 kDa unknown protein (arrow head) in *atgsnor1-3* which run at ~70 kDa after conjugation to SUMO5 was found to be deSUMOylated during the establishment of disease with *Pst*DC3000. No significant changes were seen in overall SUMO5-conjugate and free SUMO5 levels. Four-week old plants were infiltrated with virulent *Pst*DC3000 suspension set at 10^6 cfu/ml. Total protein extracts were separated by SDS-PAGE and subjected to immunoblot analysis using antibody specific to SUMO5. Coomassie stained gels show equal protein loading.

These data suggest a minor role of *S*-nitrosylation in modulating SUMO5 modification as the level of SUMO5 conjugation to most of the target proteins remained unchanged in both genotypes during the defence response. Despite the presence of sufficient free SUMO5 levels, the level of conjugated proteins remain unaltered suggesting SUMO5 modification is not induced during the establishment of defence response. This suggests increased SNO levels specifically regulate SUMO1/2-conjugation rather than mediating a general effect on the SUMOylation process.

4.4 Discussion

Rapid generation of ROIs along with NO production is an important outcome of a host-microbe interaction which plays a pivotal role in plant defence activation. The role of NO in several defence signalling pathways has been well established in the literature including SA and JA accumulation, ethylene production and defence gene activation (Huang et al., 2004; Grun et al., 2006; Mur et al., 2008). Furthermore, a balance between ROS and RNS production is critical for normal defence activation and cell death after pathogen recognition.

NO meets its signalling functions in part through *S*-nitrosylation, which is a redox based post-translational modification of cellular proteins characterised by the addition of an NO moiety, derived from different RNS (NO_x, N_2O_3, $ONOO^-$), to the target Cys thiol forming an *S*-nitrosothiol (SNO) (Stamler et al., 1992; Hess et al., 2001; Wang et al., 2006). NO is derived from its global reservoir GSNO which is synthesized by an O_2 dependent reaction of NO with glutathione (Gaston et al., 1993; Singh et al., 1996; Jaffrey et al., 2001). GSNO homeostasis is critical for normal cellular affairs, and this redox tone is regulated by GSNOR – a conserved enzyme from bacterial to mammals regulating cellular SNO levels. The absence of GSNOR function leads to the elevated global SNO formation and compromised resistance in plants and animals in addition to several development defects (Liu et al., 2004; Feechan et al., 2005; Wang et al., 2006; Lima et al., 2009).

Here we report a previously unknown regulation of protein SUMOylation by *S*-nitrosylation in plants. We examined global SUMO1/2, SUMO3 and

SUMO5 levels in unchallenged wild-type and *atgsnor1-3* plants and also compared them in compatible and incompatible pathogen interactions. We found that all SUMO1/2, SUMO3 and SUMO5 behave differently in different pathogen interactions. SUMO1/2 conjugation together with more protein *S*-nitrosylation was found critical during the establishment of disease as well as the defence response. We have demonstrated that *P. syringae* is capable of manipulating the host protein SUMOylation levels to cause infection while increased SUMOylation is an important outcome during the defence response displayed by the host plant after AvrB recognition.

An increase in SUMO1/2 conjugation and free SUMO accumulation in *atgsnor1-3* plants during the defence response and a sharp decrease after *Pst*DC3000 colonization suggests that *S*-nitrosylation plays an important regulatory role in protein SUMOylation/deSUMOylation after *Pst* infection. As SUMOylation is a multistep process; we hypothesize that SUMO enzymes being rich in Cys residues are the potential targets of *S*-nitrosylation and this may significantly change the dynamics of protein SUMOylation *in vivo* before and after pathogen challenge. High basal levels of HMW SUMO1/2-conjugates in *atgsnor1-3* plants compared to wild-type also support this hypothesis. Further, these data suggests that virulent pathogens have devised strategies to deSUMOylate proteins and mimic host defences. In contrast, pathogen recognition leading to defence activation and cell death results in elevated SUMOylation levels. Importantly, this modulation is *At*GSNOR-dependent and was recorded only in loss of *At*GSNOR function plants and not in the wild-type.

SUMO3 also seems to play an important role during pathogenesis and plant defence but its conjugation was found to be independent of *S*-nitrosylation. An instant SUMOylation of a ~10 kDa unknown protein both in wild-type and *atgsnor1-3* mutant plants following AvrB recognition and deSUMOylation during the establishment of *Pst*DC3000 infection suggests that virulent DC3000 might deSUMOylate host proteins to aid pathogenesis. Another possibility could be that this ~10 kDa protein in SUMO3-modified form is highly expressed after AvrB recognition and there is a loss of the protein during the establishment of disease with *Pst*DC3000. In AvrB-triggered defence responses, the same target is extensively SUMOylated portraying a picture similar to SUMO1/2 conjugation/deconjugation after pathogen challenge. A cyclic SUMOylation/deSUMOylation of a ~18 kDa protein was also observed in incompatible pathogen interactions with *Pst*DC3000(*avrB*). Nevertheless, the dynamics remained more or less the same in both wild-type and *atgsnor1-3* plants.

It can be argued that the ~10 kDa protein which is a SUMO3 target during both AvrB recognition and *Pst*DC3000 colonisation in wild-type and *atgsnor1-3* plants may be a diSUMO3-conjugate. However, this is unlikely because only SUMO1 and 2 can form poly-SUMO chains. The absence of any SUMOylation motif within SUMO3 makes it incapable of forming a di-SUMO3-conjugate (Colby et al, 2006; Budhiraja et al., 2009). Therefore, this unknown ~10 kDa protein is a significant SUMO3 target during the establishment of disease resistance and might have an important regulatory role in the defence response.

Furthermore, changes in SUMO5-conjugates seem to be less important compared to SUMO1/2 and SUMO3 during the defence and disease response after *Pst* challenge.

It was previously reported that tight regulation of protein SUMOylation is essential for the activation of effective defence responses. Both overexpressing and knockdown SUMO1/2 lines show constitutive expression of SA dependent defence genes and offer increased resistance against *Pst*DC3000 and delayed HR (van den Burg et al., 2010). We have demonstrated that *S*-nitrosylation plays an important regulatory function in modulating SUMOylation both in the presence and absence of pathogens. Moreover, virulent *Pst*DC3000 might decrease SUMOylation levels in order to aid pathogenicity. In contrast, plants respond to avirulent *Pst*DC3000 by increased SUMOylation of target proteins, which may help combat pathogen ingress. Similar findings have also been reported recently in *Listeria* infection experiments in humans where reduction in SUMOylation levels were found critical for bacterial infection and overexpressing SUMO1 or 2 significantly reduced the virulence of this bacterium (Ribet et al., 2010).

Chapter 5

5 SUMO enzymes are *S*-nitrosylated *in vitro*

5.1 Background

The coupling of an NO molecule to the reactive cysteine thiol forming an *S*-nitrosothiol is an important post-translational modification (PTM) of proteins orchestrating a variety of cellular functions. Several hundred proteins have been identified as targets of *S*-nitrosylation so far and the functional attributes of this redox-based PTM has been associated with many cellular activities. Nevertheless, the complexity of these redox based cellular signals and the transient and reversible nature of this modification remain a major challenge in demonstrating its regulatory function. Growing evidence suggest that NO plays essential signalling roles in plants including seed germination, metabolism, development, reproduction and biotic and abiotic stresses (Delledonne et al., 1998; Durner et al., 1998; Beligni and Lamattina, 2000; Lamattina et al., 2003; He et al., 2004; Besson-Bard et al., 2008; Hong et al., 2008). Furthermore, NO is regarded as a key signalling molecule during pathogen invasion and is required for SA and JA signalling, ethylene production and defence genes activation (Huang et al., 2004; Mur et al., 2008).

S-nitrosylation mainly occurs at one or a few redox sensitive Cys residue within proteins. The NO moiety required for *S*-nitrosylation may be gained from different NO-derived adducts collectively known as reactive nitrogen species (RNS). Yet, GSNO acts as a global reservoir and source of free radical NO which

is derived by its homolytic cleavage in an enzymatic reaction facilitated by GSNOR. Hence, GSNOR is a key enzyme responsible for regulating both GSNO level and SNO formation. For this reason, GSNO is the most widely used NO donor both *in vivo* and *in vitro S*-nitrosylation assays (Singh et al., 1996; Inoue et al., 1999; Jaffrey et al., 2001). Equilibrium between GSNO homolysis and SNOs is critical for normal cellular functions and is facilitated by the redox buffering properties of reduced glutathione (GSH) in the reducing environment of eukaryotic cytoplasm where a GSH/GSSG ratio of 100/1 is maintained (Walsh, 2006). A change in the cellular redox status and the engagement of the nitrosative burst is a major consequence of any pathogen ingress both in animals and plants, which leads to GSH conversion to GSNO and the orchestration of a variety of signalling reactions (Eu et al., 2000; Gaston, 2003; Romero-Puertas et al., 2008).

Attachment of an NO moiety to Cys residues is a highly selective process influenced by many physical and biochemical factors. These include protein structure, hydrophobicity of Cys surrounding proximate residues, thiol pKa and presence of metal ions like Mg^{2+} or Ca^{2+}. Interestingly, *S*-nitrosylation does not seem to be dependent upon the total number of Cys present in a protein and the choice of Cys residue(s) undergoing *S*-nitrosylation may vary under different circumstances (Lai et al., 2001; Sun et al., 2001; Wang et al., 2006; Marino and Gladyshev, 2009). A small family of enzymes known as thioredoxins (TRX) catalyse SNO decomposition making *S*-nitrosylation a reversible process (Benhar et al., 2008; Tada et al., 2008; Benhar et al., 2009).

Cys residues are regarded as having high functional significance in proteins compared to other amino acid as they are critical for protein folding and tertiary structure, are central to many enzyme active sites, bind metals and are subjected to PTM (Fomenko et al., 2007). In the previous section, it has been established that *S*-nitrosylation plays an important role in regulating protein SUMOylation *in vivo*. It was hypothesized that SUMO enzymes with conserved Cys residues could be targets of *S*-nitrosylation. To test this hypothesis, the biotin switch technique was employed in which *S*-nitrosylated Cys residues are labelled with biotin-HPDP (N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide). Utilising this approach, SUMO enzymes were found to be *S*-nitrosylated *in vitro* following exposure to given concentrations of NO donors (GSNO or CysNO). Also, the Cys targets of *S*-nitrosylation were identified by liquid chromatography (LC) coupled with mass spectrometry (MS) and site-directed mutagenesis. Furthermore, *S*-nitrosylation of SUMO enzymes was found to be NO donor concentration dependent and this modification was reversed by the addition of the reducing agent, dithiothreitol (DTT).

5.2 *In vitro S*-nitrosylation of SUMO enzymes

Recombinant SUMO E1 (SAE1a, SAE1b and SAE2) and E2 (SCE1) enzymes were expressed in *E. coli* cell cultures and purified under native conditions by affinity chromatography. In order to test if these enzymes are *S*-nitrosylated *in vitro*, the enzymes were incubated with 200 μM GSNO under dark and the biotin switch assay was performed to monitor SNO formation. The control samples were treated with reduced glutathione (GSH) which is devoid of an NO

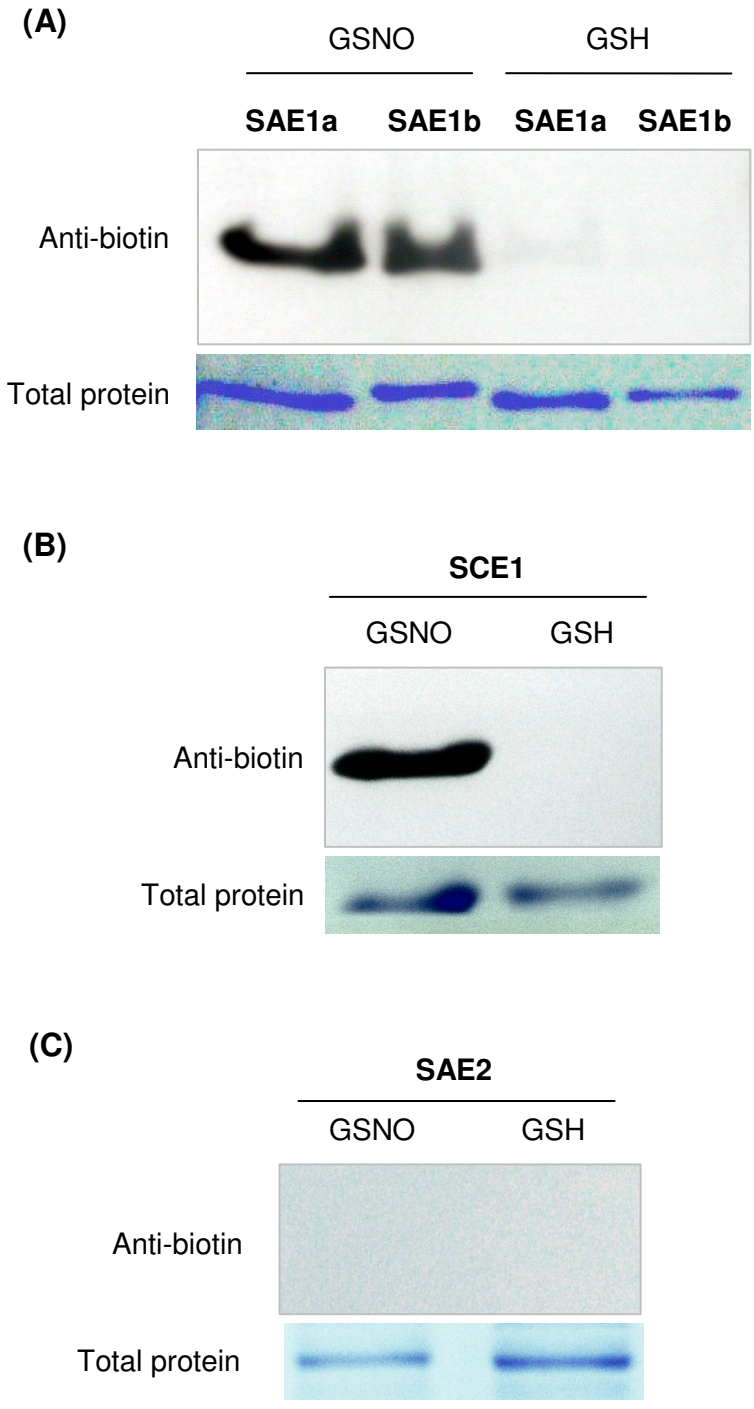

Figure 5-1 SUMO enzymes are *S*-nitrosylated *in vitro*.

(A) SAE1a and SAE1b and **(B)** SCE1 and readily *S*-nitrosylated *in vitro* but **(C)** SAE2 is not. Purified recombinant SUMO enzymes (50 µg each) were incubated with 200 µM GSNO or GSH and biotin switch assays were performed. The samples were subjected to immunoblot analysis using anti-biotin antibody. Coomassie blue stained gels indicate equal protein loading.

moiety, hence can not donate NO group to the reactive Cys-thiols. SDS-PAGE followed by western blot analysis was carried out to mark *S*-nitrosylated enzymes using anti-biotin antibody. It was found that SAE1a, SAE1b and SCE1 are readily *S*-nitrosylated *in vitro* (Fig 5-1, A and B). The absence of any signal on the autoradiogram for SAE2 showed that no Cys residue is targeted by NO, suggesting that this enzyme is not modified by *S*-nitrosylation (Fig 5-1, C).

5.3 *S*-nitrosylation of SUMO enzymes is GSNO concentration dependent and DTT reversible

In order to test if *S*-nitrosylation of SUMO enzymes is GSNO concentration dependent, the protein samples were incubated with different concentrations of GSNO in the dark followed by a biotin switch assay and western blot analyses. In order to discriminate between *S*-nitrosylation and *S*-glutathionylation, *S*-nitrosocysteine (CysNO) was included in the assay which supports *S*-nitrosylation but not *S*-glutathionylation. Since, *S*-nitrosylation is a reversible process and SNOs are readily decomposed by the addition of a reducing agent, the presence of an SNO group was verified by adding 20 mM dithiothreitol (DTT) to the samples after incubation with GSNO. The negative control was 500 μM GSH added to the samples replacing GSNO. Being devoid of an NO moiety, GSH does not donate an NO group to the reactive Cys-thiols and thus no *S*-nitrosylation is expected. This negative control is indispensable because a false signal could be obtained due to insufficient blocking of non reactive Cys with MMTS.

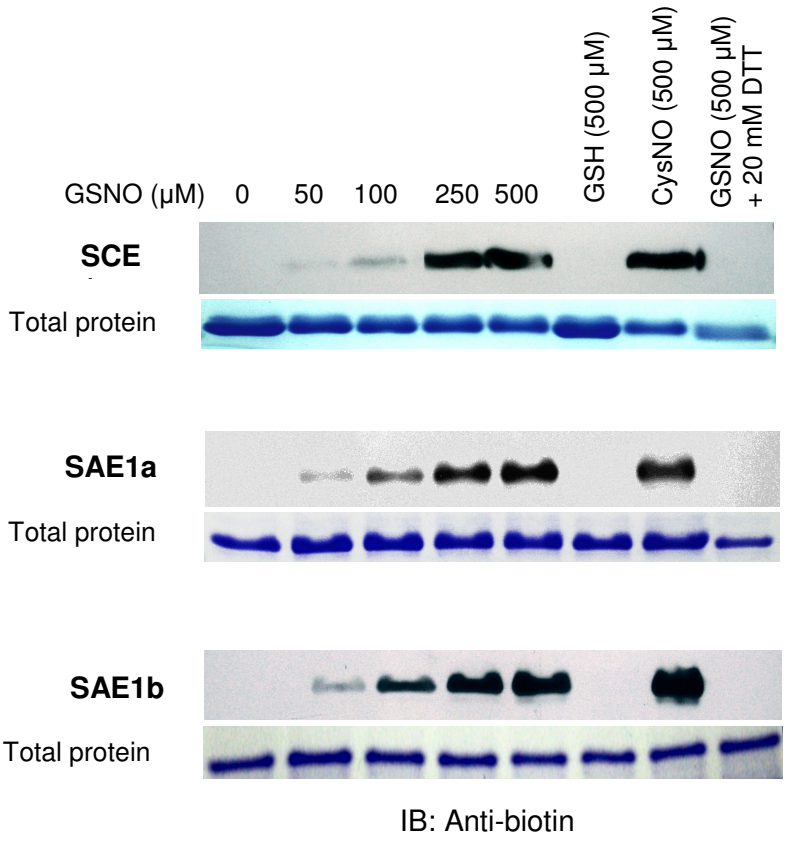

Figure 5-2 *S*-nitrosylation of SUMO enzymes is GSNO concentration dependent and DTT reversible.

A substantial increase in signal intensity was observed with an increase in GSNO concentrations reaching a maximum of 500 µM. Faint signals were obtained at 50 µM GSNO which was the lowest concentration used. SAE1a, SAE1b and SCE1 are readily *S*-nitrosylated by CysNO. No signal was seen in GSH negative control lanes and in the first lanes without GSNO. Adding 20 mM DTT reduced the SNOs, therefore, yielding no signal in the last lane. SUMO enzymes were subjected to a biotin switch assay after incubation with the stated concentrations of GSNO, GSH, CysNO or DTT followed by SDS-PAGE and western blot analyses. Immunodetection was made by using an anti-biotin antibody. Coomassie blue stained gels indicate equal loading.

It was found that *S*-nitrosylation of SUMO enzymes is GSNO concentration dependent (Fig 5-2). The signal intensity substantially increased with an increase in GSNO concentration, reaching a maximum of 500 μM. As expected, no signal was obtained in GSH control lanes while a strong signal was obtained by incubating SUMO enzymes with 500 μM CysNO (Fig 5-2). This verifies the *S*-nitrosylation of SUMO enzymes. Moreover, CysNO could be used as an inexpensive NO donor replacing GSNO which is costly and more labile. Since, *S*-nitrosylation is a reversible process; the SNO groups formed within these enzymes were found readily reversible by the addition of 20 mM DTT (Fig 5-2).

5.4 Identification of *S*-nitrosylation sites within SUMO enzymes

After it was established that SAE1a, SAE1b and SCE1 are *S*-nitrosylated *in vitro*, liquid chromatography coupled with mass spectrometry (LC-MS/MS) was carried out to identify the Cys targets of *S*-nitrosylation in these enzymes. Since SNOs are very labile and sensitive to heat or light treatment, LC-MS/MS analysis was carried out on biotinylated peptides where the NO moiety was replaced with biotin-HPDP which makes a very stable covalent bond with Cys-thiols. The biotinylated peptides were digested with trypsin or proteinase K or both. All Cys were detected in LC-MS/MS and the spectra acquired were analysed for the specific peptides containing biotin-HPDP. Also, MS^3 was carried out to increase the confidence of detection. Site-directed mutagenesis of single Cys residues was also undertaken for SCE1 where four Cys residues were individually replaced with Ser and mutant recombinant proteins were assayed

using the biotin switch method for the presence or absence of an SNO signal after GSNO treatment.

5.4.1 LC-MS/MS revealed Cys^{139} in SCE1 is the target of *S*-nitrosylation

It was found that only Cys^{139} in SCE1 is targeted by *S*-nitrosylation and not Cys^{44}, Cys^{76} or Cys^{94} (Fig 5-6, C). The LC-MS/MS analysis was based on two independent digestions with trypsin (Fig 5-3) and proteinase K (Fig 5-4). The protein coverage remained 85% and 82% for tryptic and proteinase K digest, respectively, while all Cys were detected after both digests. The detected Cys were potentially modified by either a methylthiol group or biotin-HPDP.

The MS^2 spectra of a 3^+ peptide at 1453.3680 amu was obtained after a tryptic digest of biotinylated samples containing 8ug/ul protein with the sequence assignment of QILVGIQDLLDTPNPADPAQTDGYHLF**C**QDPVEYK (Fig 5-3). Cys^{139} was found harbouring biotin-HPDP and the mascot score for the assignment was 102 with the error mass of 0.0078 Da. Three-stage mass spectrometry (MS^3) was also performed to increase the confidence in assigning sequence match probabilities. After proteinase K digest, MS^2 spectra of two isoforms of the same peptide **C**QDPVEYKKR was obtained from biotinylated samples (Fig 5-4, B). Again Cys^{139} was found labelled with a biotin-HPDP group generating a 3^+ charged peptide at 565.2794 amu (Fig 5-4 B). MS^3 was performed at 460.4 amu (Fig 5-4, D) which supported the sequence assignment for MS^2. In the biotin switch assay, not all the reactive Cys receive NO moiety after GSNO incubation. Thus, some Cys^{139} residues were also detected harbouring a methyl

group (Fig 5-4 A) as Cys residues not undergoing *S*-nitrosylation are subsequently blocked with a methyl group by treating with MMTS.

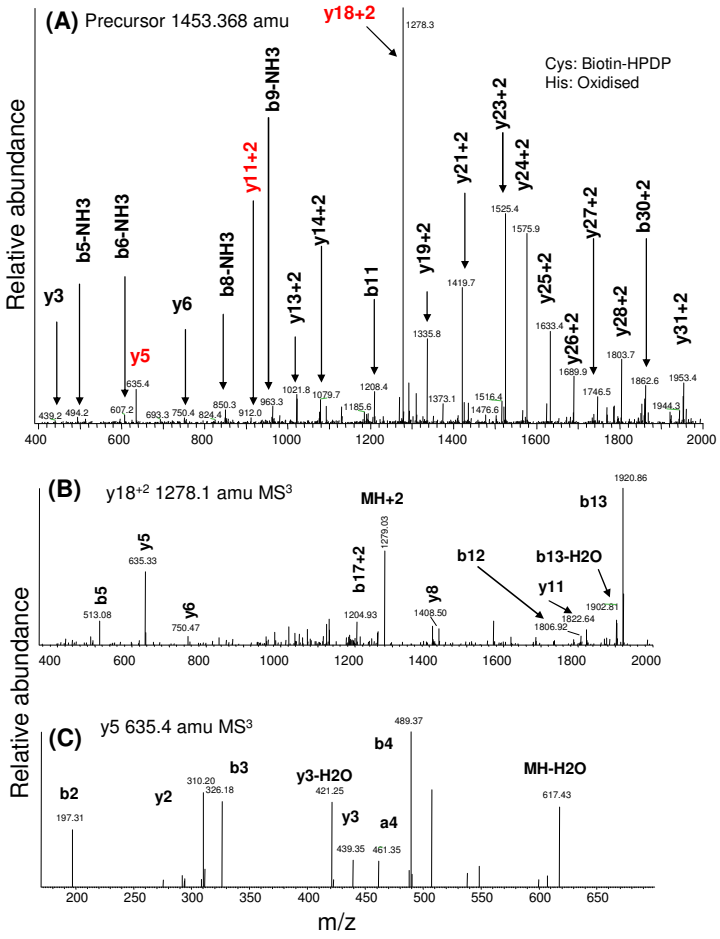

Figure 5-3 LC-MS/MS analysis after tryptic digest revealed Cys^{139} in SCE1 is *S*-nitrosylated.

MS/MS spectra obtained after tryptic digest. Recombinant SCE1 samples were incubated with 200 µM GSNO and biotinylated before tryptic digest and LC-MS/MS analysis. **(A)** MS^2 spectra of a 3$^+$ peptide at 1453.3680 amu was obtained after a tryptic digest of biotinylated samples containing 8ug/ul protein. The sequence assignment was QILVGIQDLLDTPNPADPAQTDGYHLFCQDPVEYK with the Cys^{139} blocked with biotin-HPDP. The mascot score for the assignment was 102 and the error mass was 0.0078 Da. The fragmentation pattern assignment was also shown and 2 selected MS^3 were also performed on the specific fragment, **(B)** on y18^{+2} at 1278.1 amu, and **(C)** y5 at 635.4 amu. The MS^3 assignment was performed manually without the use of MASCOT program.

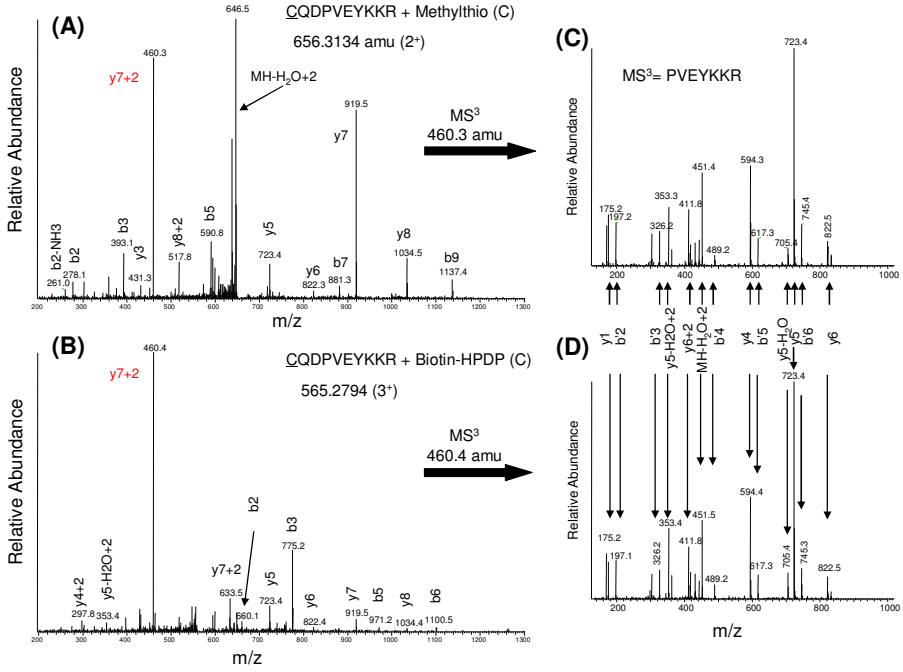

Figure 5-4 LC-MS/MS analysis after proteinase K digest showed Cys^{139} in SCE1 is *S*-nitrosylated.

MS/MS spectra obtained after proteinase K digest. Recombinant SCE1 samples were incubated with 200 µM GSNO and biotinylated before proteinase K digest and LC-MS/MS analysis. **(A)** MS^2 spectra of two isoforms of the same peptide CQDPVEYKKR obtained after proteinase K digest from biotinylated samples. The Cys^{139} is blocked with a methylthiol group generating a 2^+ peptide at 656.31 amu. **(B)** The evaluated peptide assignment is the same but with a cysteine blocked with a biotin-HPDP group generating a 3^+ charged peptide at 565.2794 amu. Both peptides shared similar y ion including the intense N-terminal proline fragment y7+2 at 460.3 amu. **(C)** and **(D)** MS^3 performed on two of these fragments at 460.3 amu generated similar fragments which support the sequence assignment done for both MS^2 and MS^3.

All four Cys in SCE1 were detected by LC-MS/MS and three of them were found to be in the methylated form, only Cys^{139} was found labelled with biotin-HPDP and this was independently confirmed by both proteases digestions and subsequent LC-MS/MS analysis. It was also noted that that peptide

modification by the large molecule biotin-HPDP does not alter the peptide elution pattern significantly.

5.4.2 SCE1 C139S mutant is not *S*-nitrosylated *in vitro*

SCE1 has four cysteine residues (Fig 5-6, C), hence, four mutant SCE1 were generated where each Cys residue was replaced with a Ser by carrying out *in vitro* site directed mutagenesis. The mutant enzymes were incubated with 100 µM GSNO or its reduced form GSH. The biotin switch assay was performed to monitor the formation of SNOs. The absence of a signal in SCE1 C139S mutant confirmed that *S*-nitrosylation of SCE1 takes place at Cys^{139} only (Fig 5-5). Mutation of Cys^{44}, Cys^{76} or Cys^{94} did not abolish *S*-nitrosylation of SCE1.

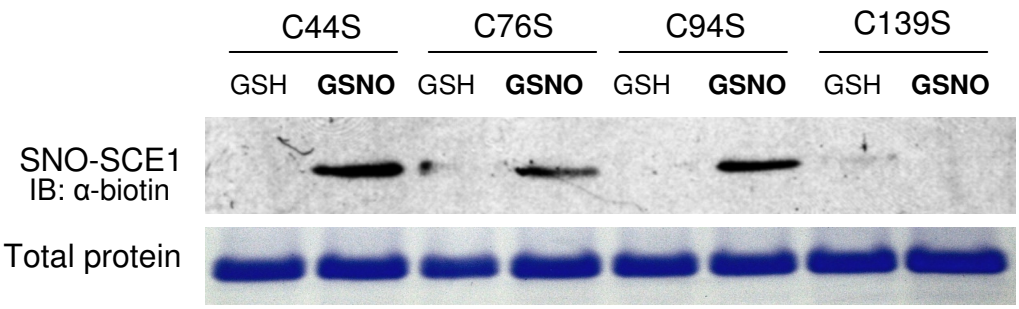

Figure 5-5 Mutation in Cys^{139} precludes *S*-nitrosylation of SCE1.
Mutant SCE1 protein samples were incubated with 100 µM GSNO or GSH and subjected to the biotin switch assay followed by western blot analysis using anti-biotin antibody. The absence of a signal in the C139S mutant of SCE1 indicates that Cys^{139} is the target of *S*-nitrosylation. Coomassie blue stained gel showing total protein indicates equal loading.

5.4.3 LC-MS/MS revealed three Cys are *S*-nitrosylated in SAE1a

SAE1a has nine cysteine residues (Fig 5-6) which are all highly conserved in its homologue SAE1b. So we tested only if SAE1a is *S*-nitrosylated *in vitro*. The SAE1a samples were treated with 200 mM GSNO and biotinylated before being subjected to tryptic digestion and LC-MS/MS analysis. It was revealed that out of nine Cys residues Cys^{93}, Cys^{158} and Cys^{231} are the targets of *S*-nitrosylation *in vitro* (Fig 5-6). The protein coverage was 60% and the peptides harbouring biotin-HPDP are given below with the predicted Cys residues circled.

VAFYTVDCR.D + Biotin-HPDP

TVAEICSDSLK.D + Biotin-HPDP

HGECSLLDLAR.V + Biotin-HPDP

```
        10         20         30         40         50         60
MHGEELTEQE TALYDRQIRV WGANAQRRLT KAHILVSGIK GTVAEFCKNI VLAGVGSVTL
        70         80         90        100        110        120
MDDRLANMEA LNANFLIPPD ENVYSGKTVA EICSDSLKDF NPMVRVSVEK GDLSMLGTDF
       130        140        150        160        170        180
FEQFDVVVIG YGSRATKKYV NEKCRKLKKR VAFYTVDCRD SCGEIFVDLQ DYKYTKKKLE
       190        200        210        220        230        240
EMVECELNFP SFQEAISVPW KPIPRRTAKL YFAMRVIEVF EESEGRKHGE CSLLDLARVL
       250        260        270        280        290        300
EIKKQLCEAN SVSESHIPDI LLERLITGTT EFPPVCAIVG GILAQEVIKA VSGKGDPLKN
       310        320
FFYYDGEDGK GVMEDISDSF TSRSHHHHHH
```

Figure 5-6 Amino acid sequence of SAE1a showing *S*-nitrosylated Cys.
SAE1a has nine Cys residues (bold/highlighted). LC-MS/MS analysis revealed three Cys residues (Cys^{93}, Cys^{158} and Cys^{231} shown by empty circles) are modified by *S*-nitrosylation.

5.5 Structural attributes of *Arabidopsis* SCE1

The 3D molecular structures of proteins give valuable information about the protein functional attributes and modulation by any PTM. To gain a better insight into *S*-nitrosylation of SCE1, structural modelling of this protein was undertaken. As there is no 3D structure available for *At*SCE1, homology modelling was carried out on Phyre web server (www.sbg.bio.ic.ac.uk/~phyre/) using protein sequence information. The predicted structure was threaded onto a known structure of human SCE (*Hs*Ubc9). The amino acid sequence alignment of both enzymes showed high homology with all four Cys and the active site highly conserved (Fig 5-8).

The *At*SCE1 modelled structure was threaded over the 3D crystal structure of *Hs*Ubc9 using PyMOL™ version 0.99 and the two structures were found to be almost identical (Fig 5-6, A). The 3D surface structure of *At*SCE1 revealed Cys^{139} as solvent exposed while Cys^{94} which is the key Cys for SUMO conjugation is relatively embedded though visible through the surface (Fig 5-6, C). The distance between these two Cys was measured as 16.13 Å and these Cys in *At*SCE1 were found at the same locations as Cys^{94} and Cys^{139} in *Hs*Ubc9 when superimposed (Fig 5-6, D and E), suggesting a very high conservation not only of the primary sequence but also of the molecular structure.

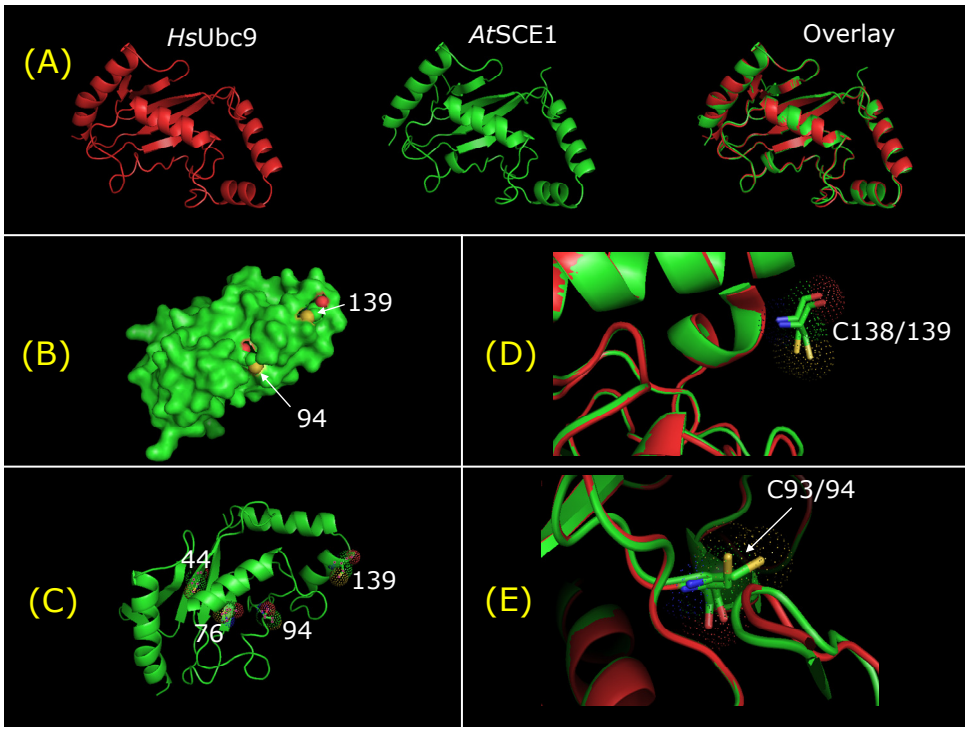

Figure 5-7 3D structural attributes of *Arabidopsis* SCE1.

Structural modelling of *At*SCE1 was carried out on the basis of its protein sequence using Phyre online server with 100% estimated precision. **(A)** Structure of *Hs*Ubc9 and *At*SCE1 and overlay of both showing very high structural identity. **(B)** Surface view of *At*SCE1; the arrow heads are showing solvent exposed Cys^{139} while Cys^{94}, though visible, is surrounded by the active site pocket. **(C)** Ribbon representation of *At*SCE1 model showing the placement of all four Cys. **(D)** Positions of *At*SCE1 Cys^{139} and *Hs*Ubc9 Cys^{138} and **(E)** *At*SCE1 Cys^{94} and *Hs*Ubc9 Cys^{93} within the respective enzymes superimposed on each other.

Figure 5-8 Sequence alignment of *Arabidopsis* and human SCE.

The amino acid sequence alignment of *At*SCE1 and *Hs*Ubc9 was generated online by using T-Coffee multiple sequence alignment tool (www.igs.cnrs-mrs.fr). Sequence alignment is shaded in different colours indicating high, medium or low homology given in the alignment key. High amino acid sequence identity is present between enzymes from two different organisms while all four Cys, shown by their respective numbers, were found highly conserved. $Cys^{93/94}$ which is the key cysteine for SUMO conjugation is present in the conserved active site shown by an empty box. Asterisks indicate the identical residues while colons and dots indicate strong and week conservation, respectively.

5.6 Discussion

Generation of NO during the plant defence response and its covalent attachment to Cys residues of proteins is regarded as a fundamental signalling mechanism dictating protein function. In the previous section of this dissertation, *in vivo* data was presented to support the hypothesis that *S*-nitrosylation is an important regulator of SUMOylation before and after pathogen invasion. In this chapter it has been shown that SUMO enzymes (SAE1a and SCE) are the direct

targets of *S*-nitrosylation. Further, the *S*-nitrosylation of SUMO enzymes is GSNO concentration dependent, and is reversible by the DTT treatment. Moreover, SUMO enzymes are readily *S*-nitrosylated by CysNO.

LC-MS/MS analyses were performed to identify Cys targets of *S*-nitrosylation after SUMO enzymes were treated with GSNO. As NO attachment to Cys-thiols is very labile and the SNOs are readily decomposed by exposure to light or heat, chemical treatment or even a change in the pH. To deal with this problem, the protein SNOs were replaced with a very stable biotin-HPDP and subsequently analysed. It was found that Cys^{139} in SCE1 is the target of *S*-nitrosylation. The *S*-nitrosylation of Cys^{139} was further verified by site directed mutagenesis experiments where all four Cys present in SCE1 were replaced with Ser followed by the biotin switch assay. The C139S mutant SCE1 was not *S*-nitrosylated although the remaining three Cys were present suggesting Cys^{139} is the sole target of *S*-nitrosylation in SCE1.

It has been previously described that Cys motifs targeted by NO may be pointed outwards and are solvent accessible (Marino and Gladyshev, 2010). In order to get a structural insight into SCE1 Cys^{139} *S*-nitrosylation, computational methods were used to simulate the 3D structure as there was no structure available for SCE1. The hypothetical molecular structure was obtained by template base homology modelling which relies on the known 3D structures of a homologue which, in the present case, was *Hs*Ubc9. The structural analysis revealed that SCE1 Cys^{139} is relatively solvent exposed which increased its likelihood of being *S*-nitrosylated. Further, it was also found that the Cys residues in SCE1/Ubc9 are

conserved in *Arabidopsis* and human as these enzymes perform similar functions in two different organisms. The conservation of Cys residues suggests their important functional role in conjugation activity of SCE1 which can be mechanistically similar in both organisms. Biotin switch assay was also carried out with SAE1a followed by LC-MS/MS which revealed three cysteines (Cys^{93}, Cys^{158} and Cys^{231}) as the targets of *S*-nitrosylation.

Taken together, these data suggest that *S*-nitrosylation of SUMO enzymes may have an important regulatory role in modulating SUMOylation and, consequently, regulating a variety of cellular functions. These findings also imply possible crosstalk between *S*-nitrosylation and SUMOylation to coordinate many cellular activities.

Some of these data contrast with a recent published report from the animal NO field (Qu et al., 2007) where Cys^{75} was identified as the target of *S*-nitrosylation in *Hs*Ubc9 after HEK293 cells expressing *Hs*Ubc9 Cys-Ser mutants were treated with 500 mM GSNO and subjected to the biotin switch assay. It can be speculated that the dynamics of *S*-nitrosylation of SCE in both organisms might be different. However, the data presented in this study was obtained by using purified recombinant protein subjected to biotin switch assay. Several other factors could possibly influence the *S*-nitrosylation pattern when HeLa cell lines having an active endogenous SUMOylation machinery are transfected with expression vectors encoding wild-type and Cys mutant proteins and directly treated with high GSNO (500 μM) concentrations (Qu et al., 2007). Moreover, we carried out two independent LC-MS/MS analyses with biotinylated samples

digested with trypsin or proteinase K which allows a direct and very precise identification of Cys targets of *S*-nitrosylation. These results were also supported by site directed mutagenesis experiments of Cys residues which further confirmed that Cys^{139} is the sole target of *S*-nitrosylation in SCE1 in *Arabidopsis*.

Chapter 6

6 *S*-nitrosylation Regulates SUMOylation *in vitro*

6.1 Background

Post-translational modification (PTM) of proteins can immensely increase the diversity of protein functions by activation/deactivation of enzymes, changing protein conformation and dictating protein localization and stability. PTMs are generally reversible in nature, except for proteolytic cleavages carried out by peptidases. *S*-nitrosylation and SUMOylation are the emerging reversible PTMs regulating a wide array of cellular process both in animals and plants. Since the discovery of SUMO in 1996 (Matunis et al., 1996), the role of SUMOylation has been associated with a variety of different cellular processes essential for the survival of living cells. There is a wealth of information available not only on the biochemical and mechanistic aspects of SUMOylation but also its targets and consequences. However, there is currently little insight into the regulation of SUMOylation and its underlying molecular mechanisms.

Since SUMOylation is a multi-step process analogous to ubiquitination, and is accomplished by the activation (E1), conjugation (E2) and ligation (E3) enzymes, there is a prospect that SUMOylation could be regulated by other PTMs, for instance, *S*-nitrosylation. Interestingly, reactive oxygen species (ROS) have already been implicated in the modulation of SUMO-conjugation/deconjugation in mammals. These data suggest that SUMOylation is induced by 100 mM H_2O_2 treatment while a severe reduction in SUMO-conjugation was observed at lower

doses of 1mM (Saitoh and Hinchey, 2000; Manza et al., 2004; Bossis and Melchior, 2006). It was suggested that H_2O_2 differentially regulates SUMO conjugation by preventing Ubc9-SUMO thioester bond formation by making a disulphide cross-link between Uba2 and Ubc9 in HeLa cells (Bossis and Melchior, 2006). Recently, a human SUMO specific protease SENP1 and yeast Ulp1 were found to be reversibly modified in response to oxidative stress induced by H_2O_2 treatment (Xu et al., 2008). Thus, changes in local redox status, perhaps mediated by NADPH-dependent oxidase (NOX) proteins, in response to given cellular cues, might contribute to the control of SUMOylation. Importantly, only a handful of Cys residues could be modified by H_2O_2 as most of the Cys residues exist in reduced form (Cys-SH) and oxidation by H_2O_2 can occur only to thiolate anions (Cys-S^-). It is highly likely that reduced Cys-thiols in SUMO enzymes are more extensively modified by *S*-nitrosylation compared to H_2O_2, which may operate to fine-tune protein SUMOylation and, eventually, many cellular functions regulated by SUMO proteins.

In the previous section the reversible oxidative modification of SUMO enzymes by *S*-nitrosylation has been unveiled and cysteines undergoing thiol modification identified. In the light of previous findings, it has been hypothesized that the modification of Cys residues in SUMO enzymes could be an important redox-based signal, fine-tuning protein SUMOylation under changing cellular redox tone, which is known to be an important outcome after attempted pathogen ingress.

We tested this hypothesis by reconstituting *Arabidopsis* SUMOylation machinery *in vitro*. The SUMOylation pathway genes (*SUMO1*, *SAE1a, SAE1b, SAE2 and SCE1*) were individually cloned in expression vectors and the proteins were over-expressed and purified from *E. coli* cell cultures under native conditions. The enzymes involved in the SUMOylation pathway were treated with different GSNO concentrations and were tested by carrying out *in vitro* SUMOylation assays to monitor any changes in SUMOylation. An already known SUMO substrate *Sc*PCNA (proliferating cell nuclear antigen) was used for this purpose. Mutant C139S SCE1 was also trialled for its SUMO-conjugation potential with or without GSNO treatment in order to determine if modification of Cys^{139} is the determinant of its conjugation ability.

6.2 Reconstitution of Protein SUMOylation *in vitro*

6.2.1 Over-expression of SUMOylation machinery in *E. coli*

In order to test the hypothesis that *S*-nitrosylation regulates *in vitro* SUMOylation, the whole *Arabidopsis* SUMOylation machinery genes (*SUMO1, SAE1a, SAE1b, SAE2, SCE1*) were individually cloned in pQE70 expression vectors and their recombinant proteins were heterologously expressed in *E. coli* strain M15[pRep4]. The model substrate *Sc*PCNA was also expressed and purified in a similar fashion to the SUMOylation machinery. Before *in vitro* SUMOylation assays were undertaken, protein expression and purification procedures were optimized by varying expression and purification conditions. The expression cultures were induced by the addition of 1 mM IPTG and the pellets were harvested for protein expression analysis.

The induced and un-induced cultures were analysed by running the samples on SDS-gels followed by staining with Coomassie blue (Fig 6-1). After extensive optimization of the procedures, high expression of SUMO1, SCE1 and *Sc*PCNA was achieved, while SAE1a and SAE1b expression remained relatively low but sufficient to purify target recombinant proteins. The expression of SAE2 was very low probably due its large size and poor solubility (Fig 6-1). However, we were able to obtain sufficient SAE2 to carry out *in vitro* assays.

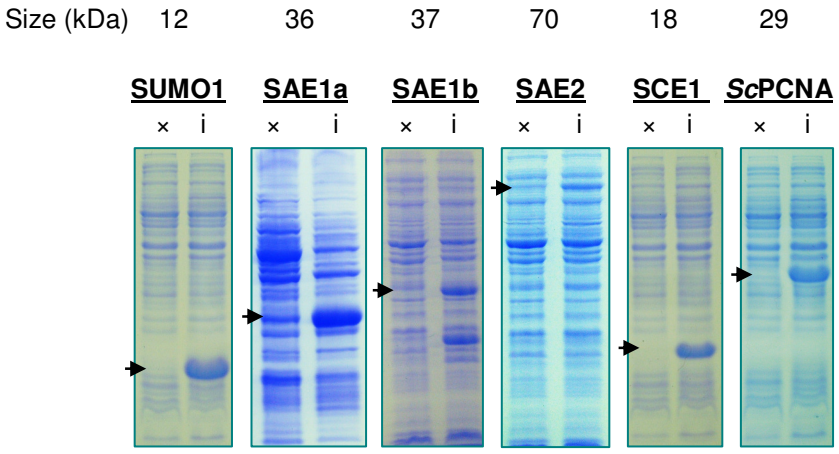

Figure 6-1 Over-expression of SUMOylation machinery in *E. coli*.

The gel pictures show the expressed protein bands of SUMOylation machinery. SUMO1, SAE1a, SAE1b, SAE2, SCE1 and *Sc*PCNA were heterologously expressed in *E. coli* expression strain M15[pRep4] by supplementing the growing cultures with 1 mM IPTG. The un-induced (x) and induced (i) cell culture pellets were lysed in 1% SDS, separated on 12% SDS gels and stained with Coomassie blue to mark the expressed protein bands shown by the arrowheads.

6.2.2 Fine-tuning the *in vitro* SUMOylation assay using *Sc*PCNA as a model substrate

The recombinant proteins for SUMO1, E1 (SAE1a, SAE1b, SAE2), E2 (SCE1) and *Sc*PCNA having C-terminal 6XHis-tag were purified by affinity chromatography and dialysed against a suitable buffer. The *in vitro* SUMOylation reaction was carried out by mixing together SUMO machinery components (8 µg SUMO1, 1 µg of SAE1a, 1 µg of SAE1b, 2 µg SAE2, 2 µg SCE1 and 4 µg *Sc*PCNA). The reaction was initiated by adding 5 mM ATP and incubated at 25 or 30 °C for 2 – 12 hrs before the SUMOylation of *Sc*PCNA was analysed by western blots. It was established that 5 hr incubation at 25 °C is the optimum condition for sufficient *in vitro* SUMOylation of *Sc*PCNA using the above protein concentrations (Fig 6-2). Moreover, doubling the amount of substrate protein did not significantly improve SUMO-conjugation hence the given protein quantities were found optimum for efficient *in vitro* SUMOylation of the target protein.

A 16 kDa size shift was observed after *Sc*PCNA was SUMOylated making a SUMO1-PCNA conjugate running at 46 kDa on SDS gels (Fig 6-2). No such band was detected in the samples without *Sc*PCNA in lane 1 (Fig 6-2, A and B). A SUMO1-SCE1 complex was detected at 32 kDa in all lanes with or without *Sc*PCNA (Fig 6-2). The uncoupled free SUMO1 was detected at 16 kDa when separated by SDS-PAGE. The mature SUMO1 with di-glycine motif exposed is originally ~10 kDa in mass, however, the SUMO1 construct was designed in such a way that it carried an N-terminal HA tag and a C-terminal 6X His-tag to facilitate purification, hence this protein was expected to run at 16 kDa.

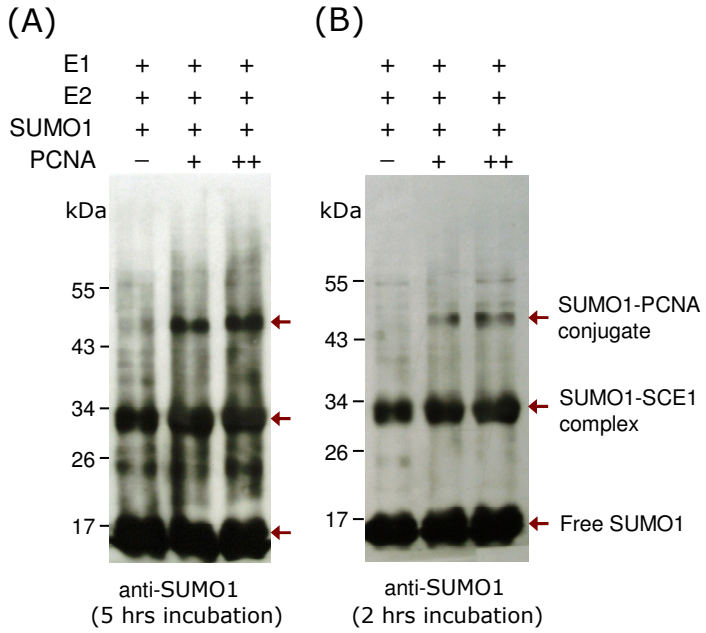

Figure 6-2 ***In vitro* SUMOylation of the model substrate *Sc*PCNA.**
Comparison between **(A)** 2 hr and **(B)** 5 hr incubation of SUMOylation reaction at 25 ℃. Free SUMO1 bearing a C-terminal 6X His-tag and an N-terminal HA-tag runs at ~16 kDa on SDS gels. SUMO1 readily forms a complex with SCE1 which runs at ~32 kDa. SUMOylation of the 30 kDa model substrate *Sc*PCNA resulted in a ~16 kDa size-shift so the SUMO1-PCNA conjugate was detected at ~ 46 kDa. No such signal was obtained in the control lanes without *Sc*PCNA. The reaction was initiated by adding ATP and incubated at 25 °C for the times shown and stopped by adding SDS sample buffer containing 25 mM DTT. Samples were boiled and separated by SDS-PAGE followed by western blot analysis using anti-SUMO1 antibody.

6.3 GSNO differentially regulates *Sc*PCNA SUMOylation *in vitro*

The *S*-nitrosylation of SUMO enzymes suggests that SUMOylation may be directly affected by GSNO treatment. To test this hypothesis, an *in vitro* SUMOylation assay was set up in which SUMO enzymes (E1 and E2) taking part in the SUMOylation pathway were incubated with different GSNO concentrations

for 20 min in the dark before these were added to the reaction mixture containing *Sc*PCNA which served as a model substrate. The reaction was triggered by adding 5 mM ATP and was allowed to proceed for 5 hrs at 25 °C.

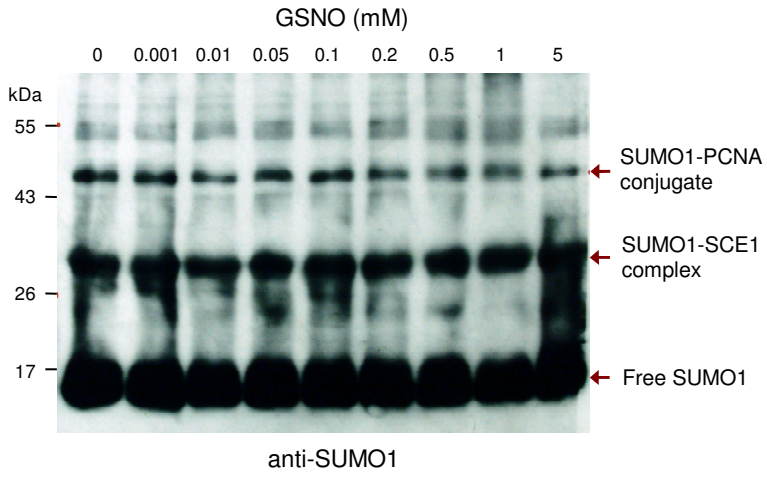

Figure 6-3 ***S*-nitrosylation of SAE1a does not effect *in vitro* SUMOylation of *Sc*PCNA.**

GSNO treated E1 enzymes do not alter *in vitro* SUMOylation of *Sc*PCNA. Recombinant E1 enzymes were treated with the given GSNO concentrations for 20 min in the dark and added to the reaction mixture containing SUMO1, SCE1 and *Sc*PCNA. The reaction was allowed to proceed for 5 hrs at 25 °C before immunoblot analysis was carried out using anti-SUMO1 antibody. SUMO1 formed a SUMO1-PCNA conjugate resulting in ~16 kDa size-shift of *Sc*PCNA eventually running at ~46 kDa. No change in *Sc*PCNA SUMOylation was detected both at low and high doses of GSNO treatment compared to untreated sample in lane 1 acting a control.

We separately investigated if *S*-nitrosylation of E1 or E2 enzymes or both have any effect on *in vitro* SUMOylation of the model substrate *Sc*PCNA. In the first step, SAE1a, SAE1b and SAE2 (E1 enzymes) were combined together in the given amounts and pre-incubated with different GSNO concentrations just before

in vitro SUMOylation assay was performed. No changes were detected in SUMO-conjugation and SUMOylation of *Sc*PCNA compared to untreated sample (Fig 6-3) suggesting that *S*-nitrosylation of SAE1a and/or SAE1b does not affect SUMOylation.

6.3.1 *S*-nitrosylation of SCE1 differentially regulates its conjugation with SUMO1 and *Sc*PCNA SUMOylation

After no change was detected in SUMOylation of *Sc*PCNA when E1 enzymes were treated with GSNO, we trialled *S*-nitrosylated SCE1 in an *in vitro* SUMOylation assay and assessed if *S*-nitrosylation of Cys^{139} in SCE1 had any regulatory role in protein SUMOylation. The SCE1 was pre-incubated with different GSNO concentrations for 20 min in the dark and was added to the reaction mixture containing SUMO1, E1 enzymes and *Sc*PCNA. The reaction was started by adding ATP and was left to proceed at 25 °C for 5 hrs before it was stopped by adding loading buffer and heating. Western blot analysis was performed using anti-SUMO1 antibody.

A significant reduction not only in *Sc*PCNA SUMOylation but also in SUMO1-SCE1 intermediates was observed at lower doses of GSNO (0.01 – 0.5 μM) (Fig 6-4). Interestingly, an increase in *Sc*PCNA SUMOylation was noticed for 5 mM GSNO treated SCE1. Also, an increase in SUMO1-SCE1 species was obvious (Fig 6-4) while the free SUMO1 levels remained constant in all the lanes indicating uniform sample loading. As SUMO1 is capable of forming poly-SUMO chains, similar reduction at low GSNO concentrations (0.01 – 0.5 μM) and an increase at high GSNO concentration was evident for diSUMO1-PCNA

conjugates (Fig 6-4). These data suggests that *S*-nitrosylation of SCE1 regulates it conjugation activity which eventually impacts *in vitro* SUMOylation of *Sc*PCNA and its ability to make intermediates with SUMO1 during the conjugation process.

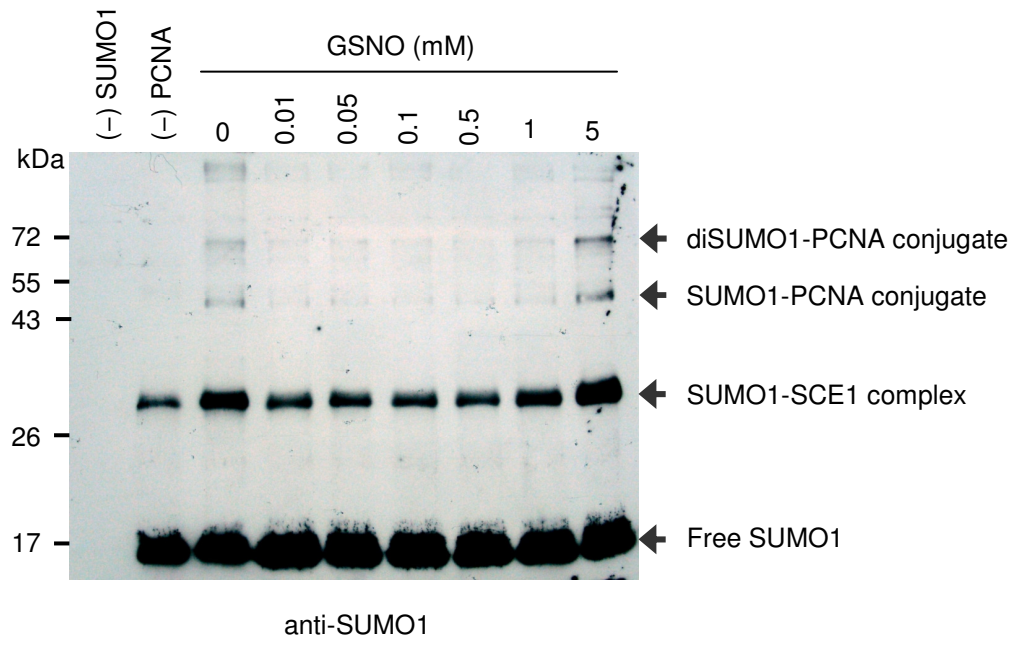

Figure 6-4 ***S*-nitrosylation differentially regulate SCE1 conjugation to SUMO1 and *Sc*PCNA SUMOylation**

A reduction in *Sc*PCNA SUMOylation was observed after SCE1 was treated with low GSNO doses (0.01 – 0.5 mM) while treatment of SCE1 with 5 mM GSNO prior to *in vitro* SUMOylation resulted in an increase in SUMOylation of *Sc*PCNA. A similar effect was observed with diSUMO1-PCNA conjugate running at ~ 62 kDa as SUMO1 can form poly-chains. A significant reduction in SUMO-conjugation to SUMO1 is apparent at lower GSNO doses while at 1 mM GSNO treatment this conjugation was restored to the control treatment level. At a relatively high GSNO concentration the level of SUMO1-SCE1 species significantly increased. Unconjugated free SUMO1 accumulated at 16 kDa indicate uniform loading.

6.3.2 Mutation of Cys^{139} in SCE1 abolishes its sensitivity to GSNO treatment

To further investigate the regulation of SUMOylation by *S*-nitrosylation of SCE1, we mutagenized Cys^{139} of SCE1 replacing it with a Ser and determined the activity of this protein in an *in vitro* SUMOylation assay after GSNO incubation. The C139S mutant and the wild-type SCE1 were incubated with different GSNO concentrations (Fig 6-5) and tested for their *in vitro* SUMOylation capacity of *Sc*PCNA.

It was found that the C139S mutant SCE1 was insensitive to GSNO treatment in the *in vitro* SUMOylation of *Sc*PCNA. In contrast, wild-type SCE1 exhibited a decrease in SUMOylation at low (0.1 and 0.5 mM) GSNO concentrations and an increase in SUMOylation at a high GSNO (2 mM) concentration, (Fig 6-5). In addition to insensitivity to GSNO treatment, reduced SUMOylation of *Sc*PCNA was detected in the C139S mutant relative to the wild-type enzyme, suggesting that the absence of Cys^{139} does not abolish enzyme activity (Fig 6-5). Thus, Cys^{139} may be important in regulating SCE1 function, implying a key role for the redox regulation of protein SUMOylation.

Unexpectedly, no changes in SUMO1-SCE1 complexes were detected (Fig 6-5). This may be due to the relative abundance of SUMO1-SCE1 species which yielded similar signal intensities after long exposure of the autoradiogram. A relatively longer period of time was desirable to sufficiently detect and mark the differences between SUMO1-PCNA conjugates, which may have otherwise blurred the differences in SUMO1-SCE1 complex formation.

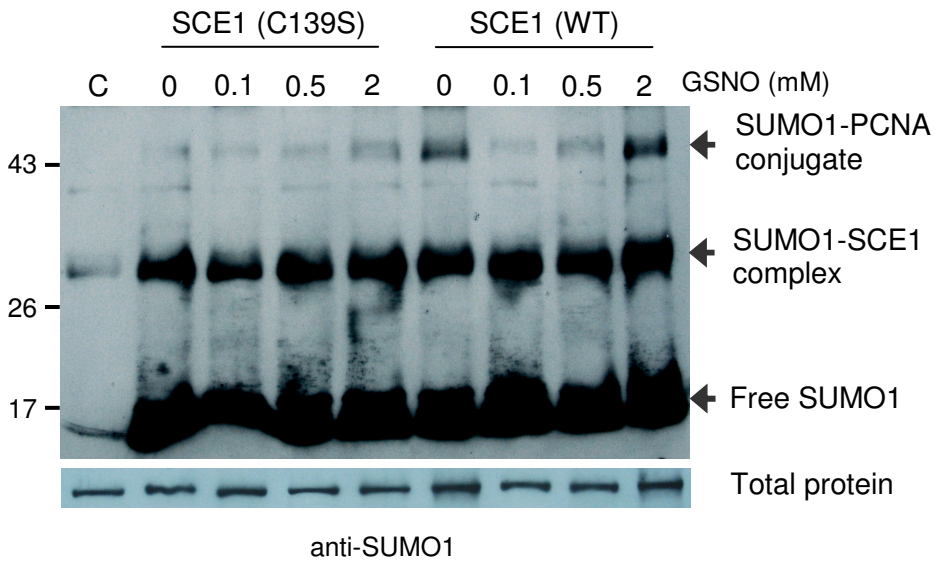

Figure 6-5 C139S mutant SCE1 is insensitive to GSNO treatment.
Mutation in Cys^{139} in SCE1 prohibited its differential regulation of SUMOylation by GSNO treatment. Wild-type and C139S mutant SCE1 were incubated with given GSNO concentrations before they were added to the *in vitro* SUMOylation assay mixture. Western blot was performed by using anti-SUMO1 antibody. Total protein shown at the bottom of the autoradiogram indicate equal loading.

6.4 Discussion

Despite intensive study of SUMOylation, the regulation of this process by PTMs remains obscure. A few reports have shown the regulation of SUMOylation by certain ROS and RNS using mammalian cell lines. For example, treating HeLa cells with 1 mM H_2O_2 completely abolished SUMO-conjugation while higher doses of H_2O_2 increased SUMOylation (Bossis and Melchior, 2006). It was indicated that low doses of H_2O_2 induce a DTT sensitive disulphide crosslink between activating and conjugating enzymes (Uba2/Ubc9) in mammalian cells. The increase in SUMO-conjugates upon high doses of H_2O_2 was suggested to be

mediated by inhibition of SENP-1 (a mammalian SUMO protease) deconjugation activity leading to more SUMO-conjugate accumulation (Bossis and Melchior, 2006). So these data relate increases in SUMOylation following redox changes to alternative mechanisms.

Similar to our findings, results have been recently published in mammalian cells showing a decrease in SUMO-conjugation after treating HeLa cells with low doses of GSNO (Qu et al., 2007). However, in contrast to our data, it was proposed that *S*-nitrosylation of Ubc9 does not interfere with its conjugation ability. Rather, the reduction in SUMO conjugates after GSNO treatment was suggested to be primarily due to degradation of Pias3 (a mammalian SUMO E3 ligase). Pias3 is present endogenously in HeLa cells and is *S*-nitrosylated upon GSNO treatment which facilitates its interaction with a ubiquitin E3 ligase Trim32 (tripartite motif-containing 32) and subsequent degradation by the proteosome (Qu et al., 2007). However, the authors failed to explain the increase in SUMO-conjugates when HeLa cells were treated with > 1 mM GSNO (Qu et al., 2007).

Unlike previous experiments carried out in HeLa cells where the endogenous SUMOylation machinery was active and many unidentified factors could have contributed to changes in SUMOylation after oxidative stress. We reconstituted the *Arabidopsis* SUMOylation machinery *in vitro* and the enzymes involved in the SUMOylation pathway were treated with different GSNO concentrations to monitor any changes in SUMOylation of a model substrate,

*Sc*PCNA. As SUMOylation does not necessarily need an E3 ligase, the *in vitro* SUMOylation assays were performed without any ligase enzyme.

Our results show that *S*-nitrosylation of SCE1 directly affects *in vitro* SUMOylation of a model substrate *Sc*PCNA. In contrast to the results presented by Qu and co-workers (Qu et al., 2007), it has been demonstrated here that *S*-nitrosylation of SCE at Cys^{139} is responsible for the differential regulation of *in vitro* SUMOylation of the model substrate which was found independent of a SUMO E3 ligase. Interestingly, *S*-nitrosylation of SCE1 inhibited SUMOylation at lower doses of GSNO (50 – 500 μM) but promoted this modification at higher dose (5 mM), suggesting a differential regulation of SUMOylation in response to a gradient of GSNO concentrations.

It might be possible that higher doses of GSNO modify additional Cys residues (e.g. Cys^{44} or Cys^{76}), which reverses the inhibitory effect of SNO formation at Cys^{139}. This may improve enzyme activity, increasing SUMO1-SCE intermediates and thereby promoting SUMOylation. Mutation of Cys^{139} rendered SCE1 insensitive to GSNO suggesting *S*-nitrosylation of redox active Cys^{139} is responsible for these effects.

7 General Discussion

SUMOylation is essential for the cell survival and individual gene knockouts for SUMO1/2, E1 and E2 enzymes are lethal (Dohmen, 2004; Saracco et al., 2007; Zhao, 2007). Since, *Arabidopsis* SUMO proteins are very similar, gene functional redundancy seems to mask any phenotype both in the presence or absence of pathogens. This was a major hurdle in using reverse genetic approach to explore the roles of SUMOylation in plant defence during the course of this study, and is a likely primary reason of slow progress made by others in this area. To deal with this problem, van den Burg and co-workers (van den Burg et al., 2010) used miRNA triggered gene silencing and generated knock-down *sumo1/2* and *sumo3* mutants. The pathogenicity tests suggested that *SUMO1/2* are critical for defence activation upstream of SA while *SUMO3* is induced during defence response and SA treatment, therefore, contributing downstream of SA. Yet, the question of how SUMOylation takes part in these responses is still open.

7.1 *S*-nitrosylation regulates protein SUMOylation in plants

SUMOylation is known to be induced by chemical and heat treatments (Kurepa et al., 2003; Chaikam and Karlson, 2010). For example, plants exposed to high temperatures, H_2O_2, canavanine or ethanol exhibited increased levels of SUMO-conjugates (Kurepa et al., 2003). However, the regulation of SUMOylation by post-translational modifications in plants is still unexplored. Here we have reported for the first time a previously unknown regulation of protein SUMOylation by *S*-nitrosylation in plants. We used *Arabidopsis* GSNOR1

(*atgrnor1-3*) loss-of-function mutant as a tool to study *in vivo* regulation of protein SUMOylation and compared the differences in SUMOylation levels with the wild-type plants before and after pathogen challenges.

Elevated endogenous levels of HMW SUMO1/2-conjugates in *atgsnor1-3* plants were recorded compared to wild-type suggesting high cellular SNOs induce SUMOylation in plants. SUMO conjugation was further increased over-time in *atgsnor1*-3 plants during the defence response against *Pst*DC3000(*avrB*). The cellular pools of unknown proteins modified by SUMO3 and SUMO5 were also increased during *Pst*DC3000(*avrB*) challenge to a lesser extent than SUMO1/2 modified proteins. In contrast, increased SUMO1/2 deconjugation occurred during *Pst*DC3000 infection in *atgsnor1-3* plants relative to the wild-type. Similar data have recently been published in *Listeria* infection experiments in mammals where a significant reduction in SUMO1/2-conjugates was recorded in infected HeLa cells while overexpressing SUMO1 and 2 contained bacterial growth (Ribet et al., 2010). Some previous reports also highlighted similar observations. The SUMO-specific protease YopJ deployed by bacterial TTSS in the host cells disrupts SUMOylation and eventually blocks MAPK signalling by inhibiting the NF-κB pathway (Orth et al., 2000; Orth, 2002). Moreover, a bacterial effector XopD was found to facilitate deSUMOylation and suppress the defence response and cell death by inhibiting SA and JA induced transcription and defence gene activation (Hotson et al., 2003; Chosed et al., 2007; Kim et al., 2008; Kay and Bonas, 2009). Likewise, a *Xanthomonas* YopJ -like effector, AvrXv4, has been shown to exhibit SUMO isopeptidase activity in plant cells which deSUMOylates host proteins to

aid pathogenicity (Innes, 2003; Roden et al., 2004). Certain viruses have also been shown to interfere with host protein SUMOylation for successful invasion (Castillo et al., 2004; Schramm and Locker, 2005; Boggio and Chiocca, 2006). Our *in vivo* data indicate that deSUMOylation of host cellular proteins by *Pst*DC3000 is *At*GSNOR1-dependent as it was observed only in loss of *At*GSNOR function plants. Taken together, these findings imply that *S*-nitrosylation plays an important regulatory function in modulating protein SUMOylation in plants both in the presence and absence of pathogens.

7.2 SUMO enzymes are *S*-nitrosylated *in vitro*

S-nitrosylation is known to regulate a variety of different regulatory proteins and enzymes, ion channels, metal and DNA binding proteins, and transcriptional factors (reviewed in Gaston et al., 2003; Hess et al., 2005; Wang et al., 2006). Since, SUMOylation is a multistep process undertaken by the active site cysteines of SUMO activating (E1) and conjugating (E2) enzymes. These enzymes form a thioester bond with glycines of SUMOs before the SUMO binds to the substrate lysine (Johnson, 2004; Lois and Lima, 2005; Olsen et al., 2010). In the light of findings in plants, we asked if the Cys residues within the SUMO enzyme are targeted by *S*-nitrosylation and whether this modification has any regulatory role in protein SUMOylation. The biotin switch assay revealed that SAE1a, SAE1b and SCE1 are modified by NO *in vitro* in a GSNO dose dependent manner. Moreover, this modification was DTT reversible and was recapitulated by replacing the NO donor (GSNO with CysNO).

The binding of NO moiety to Cys residues is an exquisitely selective process and is important to precisely convey redox-based cellular signals in order to meet different cellular demands under varying circumstances. Therefore, the identification of the Cys targeted by *S*-nitrosylation has been an important area of investigation in redox biology. We carried out LC-MS/MS analyses to identify Cys modified by NO and also carried out site directed mutagenesis of Cys residues to further elaborate our findings. It was revealed that a single Cys in SCE1 (Cys^{139}) and three Cys in SAE1a (Cys^{93}, Cys^{158} and Cys^{231}) are targeted by *S*-nitrosylation. The 3D structural modelling of SCE1 suggested that Cys^{139} is relatively solvent exposed which makes it more accessible to NO and is located 16.13 Å aside from the active site Cys (Cys^{94}).

The data presented here does not recapitulate the findings by others in mammals where Cys^{75} has been shown as the target of *S*-nitrosylation in *Hs*Ubc9 using HEK293 cell line (Qu et al., 2007). It might be possible that the modulation of SCE1/Ubc9 by *S*-nitrosylation is different in two dissimilar systems. Nevertheless, *Arabidopsis* SCE1 appeared to be highly similar in tertiary structure to human Ubc9 with all four Cys highly conserved. Both of these enzymes perform similar functions in both organisms. Moreover, the data obtained in the present study used liquid chromatography coupled with mass spectrometry to directly identify *S*-nitrosylated Cys after two independent digestions of biotinylated proteins carried out with trypsin and proteinase K. The results were also confirmed by site directed mutagenesis experiments which strengthened the argument that Cys^{139} is the sole target of *S*-nitrosylation in SCE1 in *Arabidopsis*.

7.3 *S*-nitrosylation regulates SUMOylation *in vitro*

We hypothesized if the modification of Cys residues in SUMO enzymes is an important mechanism modulating protein SUMOylation. Whilst, *S*-nitrosylation of SUMO enzymes could be an important redox-based signal, fine-tuning protein SUMOylation under changing cellular redox tone, which is an important outcome of an attempted pathogen ingress.

This hypothesis was tested in an *in vitro* SUMOylation assay by reconstituting the *Arabidopsis* SUMOylation machinery by heterologously expressing recombinant proteins in bacterial cells. The proteins were purified and were subsequently combined together for *in vitro* SUMOylation of a model substrate *Sc*PCNA. Incubating E1 enzymes with GSNO had no impact on *in vitro* SUMOylation of the model substrate *Sc*PCNA. However, a substantial reduction in *Sc*PCNA SUMOylation as well as SUMO1-SCE1 intermediates was observed when SCE1 was incubated with low doses of GSNO (50 – 500 μM). In contrast, increased activity of SCE1 rendering more SUMOylation of *Sc*PCNA and more SUMO1-SCE1 intermediates was seen when SCE1 was treated with higher doses (5 mM).

To further investigate this modulation, the C139S mutant SCE1 was generated and tested in an *in vitro* SUMOylation assay with or without GSNO treatment. The differences were compared with the wild-type SCE1 enzyme. It was found that the C139S mutant enzyme was insensitive to GSNO treatment exhibiting reduced SUMOylation of *Sc*PCNA even without GSNO incubation. This implies that Cys^{139} does not abolish enzyme activity; however, this mutation

rendered SCE1 insensitive to GSNO. Thus, *S*-nitrosylation of redox active Cys^{139} may be responsible for modifying SCE1 function. These data implies that Cys^{139} is important in modulating the transfer of SUMO1 to the target protein which was compromised in the C139S mutant, and as expected, GSNO treatment did not contribute to any change in enzyme activity because of the absence of this redox sensitive Cys.

S-nitrosylation is also known to cause moderate structural rearrangements in proteins affecting their physiochemical properties and electrostatic potential distribution, and ultimately their function (Marino and Gladyshev, 2010). The possibility that treatment of SCE1 with a higher dose of GSNO modified another Cys residue which enhanced the activity of this enzyme can be further tested. Another possibility could be that high doses of GSNO resulted in tyrosine nitration as there are six tyrosine (Tyr) residues present in SCE1, with one present in the enzyme active site, while two Tyr residues flanking Cys^{139}. Also, the nitration of other residues in SCE1 like tryptophan and/or methionine could occur in the presence of high concentrations of GSNO.

SUMOylation is an intensively studied phenomenon but its regulation by other PTMs is not well explored, except for a few studies indicating the regulation of SUMOylation by ROS and RNS in animals (Saitoh and Hinchey, 2000; Manza et al., 2004; Bossis and Melchior, 2006; Qu et al., 2007). Unlike previous experiments carried out with HeLa cells having active endogenous SUMOylation machinery, we reconstituted the *Arabidopsis* SUMOylation machinery *in vitro*. Our data suggests that *S*-nitrosylation of SCE1 is directly responsible for the

differential regulation of *in vitro* SUMOylation of the model substrate *Sc*PCNA. It may be speculated and further tested that higher doses of GSNO modify additional Cys residues (e.g. Cys^{44} or Cys^{76}) which reverses the inhibitory effect of SNO formation at Cys^{139} by improving enzyme activity thereby promoting SUMOylation. However, the insensitivity of SCE1 C139S mutant to both low and high doses of GSNO (0.1 – 2 mM) suggests that *S*-nitrosylation of the redox-active Cys^{139} is an important connection in cross-talk between two post-translational modifiers (i.e. SUMOylation and *S*-nitrosylation) in regulating protein function after pathogen invasion.

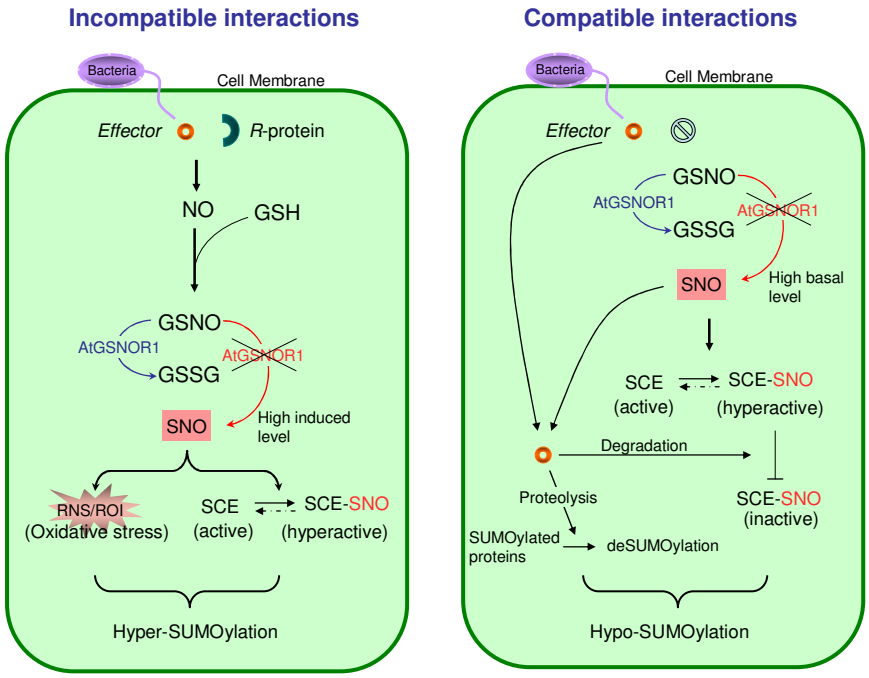

Figure 7-1 A model showing the regulation of SUMOylation by *S*-nitrosylation.

A model proposed to show how *Pst*DC3000(*avrB*) and *Pst*DC3000 subvert protein SUMOylation in plants. In an incompatible pathogen interaction, recognition of a bacterial effector by an R protein in *atgsnor1-3* plants results in more SNO formation due to ROI/RNS disequilibrium where RNS override ROIs. This results in *S*-nitrosylation of SCE1 at Cys^{139} and leads to its hyperactivation and hyper-SUMOylation of cellular proteins. In the absence of an R protein, the bacterial effector(s) may lead to proteosome independent degradation of SCE1 as in animals (Ribet et al., 2010) even when SCE1 is hyperactive due to *S*-nitrosylation. This subjugates further SUMOylation of cellular proteins. Since, certain bacterial virulence factors are also known to act as SUMO-specific proteases. These type-III effectors may cause deSUMOylation of cellular proteins which seems to be facilitated by higher cellular GSNO/SNO levels as this was only found in *atgsnor1-3* plants and not in the wild-type. This leads to hypo-SUMOylation of cellular proteins during the establishment of infection by *Pst*DC3000.

7.4 Further prospects

The work presented here has opened many avenues in redox biology where one post-translational modification system '*S*-nitrosylation' regulates another 'SUMOylation' during the establishment of disease and defence response in plants. The primary focus of this study remained on the global regulation of protein SUMOylation in plants during pathogen invasion. This study identified certain unknown proteins targeted by SUMOs during defence response which were subsequently deSUMOylated during the establishment of disease. These targeted proteins can be the important components of the defence proteome and might play important regulatory roles in defence signalling after SUMO modification. Identification of these novel targets by 2D gel electrophoresis and mass spectrometry could be informative and enhance our understanding of how SUMOylation regulates defence responses in plants.

Moreover, the biological significance of Cys^{139} modification by *S*-nitrosylation can be validated in plants which can be done by generating transgenic lines in a *sce1* mutant background expressing C139S SCE1. These lines can be the subject of pathogenicity tests using virulent and avirulent pathogens. It may also be informative to express C139S SCE1 in *atgsnor1-3 sce1* plants to determine how *S*-nitrosylation insensitive SCE1 behaves in an elevated GSNO environment after pathogen challenge. Moreover, plants containing mutations in *SUMO1* and *SUMO2* or *SUMO3* in an *atgsnor1-3* genetic background can also be generated and tested against pathogens.

The differential regulation of SUMOylation by GSNO treated SCE1 can be further explored and several different possibilities including *S*-nitrosylation at other than Cys^{139} after high GSNO doses can be examined. Further, modification of residues other than Cys e.g. tyrosine nitration can also tested by western blot analysis using antibody against nitrotyrosine and further verified by MS analysis. Gain of function tyrosine nitration in one of the several possibilities hyperactivating SCE1 undergoing 2-5 mM GSNO treatments.

Other defence related proteins can also be trialled in an *in vitro* SUMOylation assay and the targets can be further studied *in vivo* by immunoprecipitation and pull-down assays. For example, *At*GSNOR1 could also be a potential target of SUMOylation. The SUMOplot analysis program (which predicts and scores SUMOylation sites in the protein of interest) identified Lys^{162} and Lys^{191} as the targets of SUMO modification with > 90% probability, thus implying that SUMOylation might regulate *S*-nitrosylation. Similarly, other proteins important in defence signalling can also be tested this way. It is tempting to speculate that regulation of SUMOylation by *S*-nitrosylation could be important in the regulation of other defence related proteins and may be acting as a link between redox-based signals and defence activation at certain nodes of defence signalling networks. This knowledge could be very useful to better understand health and disease issues both in plants and animals.

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